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A novel strategy of controlling bovine pneumonic pasteurellosis: transfecting the upper respiratory tract of cattle with a gene coding for the antimicrobial peptide cecropin B

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A NOVEL STRATEGY OF CONTROLLING BOVINE PNEUMONIC PASTEURELLOSIS: TRANSFECTING THE UPPER RESPIRATORY TRACT OF CATTLE WITH A GENE CODING FOR THE ANTIMICROBIAL PEPTIDE CECROPIN B

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ABSTRACT

The very potent antibacterial activity of cecropin B makes it a likely candidate to prevent and/or treat *Mannheimia haemolytica* 1:A infection in the upper respiratory tract (URT) of cattle. The purpose of this study was to ascertain if the URT could be transfected with a gene coding for the antimicrobial peptide cecropin B. By transfecting cattle with a gene coding for cecropin B, this study attempted to inhibit colonization of a virulent strain of *M. haemolytica* 1:A in the URT while investigating any possible changes in the indigenous and transient nasal flora.

In this study the antibacterial efficacy of cecropin B for a virulent strain of *M. haemolytica* 1:A was determined. *In vitro* results showed that cecropin B was very effective in inhibiting this virulent strain of *M. haemolytica* 1:A within 20 minutes of incubation at 37°C. No inhibition of its activity was observed by incubating cecropin B in pooled bovine nasal secretions.

The nasal passages of calves were aerosolized with different amounts of plasmid DNA containing a gene coding for cecropin B. Results of this study show that calves transfected with 50 or 100 µg of plasmid DNA per nostril were able to express cecropin B at the mRNA and peptide level. Detection of the cecropin B gene in control calves may indicate the possibility of native bovine cecropin.

After challenge with a virulent strain of *M. haemolytica* 1:A, all calves were stressed by transportation in a crowded trailer 100 miles for 3 hours. Seven out of the 8 control calves yielded detectible levels of *M. haemolytica* 1:A in nasal aspirates throughout the weeks following challenge. All 4 calves given 25 µg of plasmid DNA per nostril and two

of the 4 calves given 50 µg of plasmid DNA per nostril yielded detectible levels of *M. haemolytica* 1:A in nasal aspirates following challenge. However, *M. haemolytica* 1:A was not detected in any calf given 100 µg of plasmid DNA per nostril. There appeared to be no change in the normal bacterial nasal flora.

INTRODUCTION

The disease known as pneumonic pasteurellosis was first described in cattle during the late 1800's (Mosier et al., 1989). Prevalence in the stockyards after shipment has earned this disease the common name 'shipping fever'. Today 'shipping fever' continues to be responsible for major economic losses in the North American beef cattle industry. More than a billion dollars are lost annually due to clinical disease and death in feedlot cattle (Brennan et al., 1998, Confer et al., 1994). Pneumonic pasteurellosis is multifactorial and complex, with stress and other environmental factors contributing to pathogenesis of the disease (Mosier et al., 1989). However, it is believed that a bacterium commonly isolated from the nasal passages of healthy cattle is a major cause of pasteurellosis (Biberstein et al., 1960). *Mannheimia haemolytica* 1:A (formerly *Pasteurella haemolytica* 1:A) produces the fibrinous pneumonia associated with the disease (Mosier et al., 1989), and is termed the major etiologic agent of bovine pneumonic pasteurellosis (Dalgleish, 1990, Frank, 1986).

While *M. haemolytica* serotype 1:A is the Gram-negative bacterium generally regarded as the cause of bovine pleuropneumonia it can be a normal resident of the bovine nasal tract. Under times of stress, *M. haemolytica* 1:A proliferates in the upper respiratory tract (URT). Stressors include transportation, crowding, irregular feeding or watering, abrupt climate change, and exposure to viral agents or a combination of the above (Frank et al., 1986, Yates, 1982). Stress induced proliferation of *M. haemolytica* 1:A following transportation is most likely the cause of pneumonic pasteurellosis in

stockyards (Frank et al., 1986, Yates, 1982). Treatment within 24 hours of shipment may be important for the protection of these cattle.

Vaccines which provide cattle with consistent immunity to pneumonic pasteurellosis are not yet available. Knowledge of *M. haemolytica's* virulence factors and the host immune response is necessary for the development of effective vaccines. Data obtained from aerosol vaccination of 205 kg calves has shown that aerosol vaccination at the level of the middle meatus enhances *M. haemolytica* 1:A clearance from the nasal passage and prevents migration of *M. haemolytica* 1:A to the lung, while vaccination at the level of the tracheal bifurcation does not affect nasal colonization of *M. haemolytica* (Brennan et al., 1998). Furthermore, vaccination at the level of the middle meatus results in the production of IgA type antibodies to various *M. haemolytica* whole cell and outer membrane antigens which can be detected in nasal secretions (Brennan et al., 1998). These studies indicate the importance of stimulating immunity to pneumonic pasteurellosis in the URT.

Since vaccines which provide cattle with consistent immunity to pneumonic pasteurellosis are not yet available, most veterinarians rely on antimicrobial agents to treat the disease. Streptomycin, tetracyclines, β-lactam antibiotics, chloramphenicol, sulfonamides, and trimethoprim-sulfonamide combinations have all been used to treat pneumonic pasteurellosis. Studies have shown *M. haemolytica* 1:A to have an increasing pattern of resistance to all of these antimicrobial agents (Hartman et al., 1993).

Public concern for the use of antibiotics in food is once again gaining momentum. Although the use of antibiotics may result in significantly harmful residue levels in food meant for human consumption, these illegal drug residues are no longer being found in

carcasses. The concern has now shifted to drug-resistant microbes. Many producers use low doses of antibiotics as growth promoters (Ferber, 2003). The result is an increased occurrence of antibiotic resistant bacteria, which may pose a threat to human health (Ferber, 2003). An effort to eliminate the routine use of antibiotics in food animal production is underway (Ferber, 2003). It may be beneficial to find novel antimicrobials to prevent and/or treat infection.

The Cecropia moth, *Hyalophora cecropia*, responds to bacterial infection with very effective antibacterial activity. The pupae synthesize several different classes of antibacterial proteins (Van Hofsten et al., 1985, Xanthopoulos et al., 1988). The most potent of these are the cecropins, specifically cecropins A, B, C, D, E, and F (Boman et al., 1987). Cecropins are small strongly basic proteins which have a broad spectrum of bactericidal activity (Steiner et al., 1988), but are unable to disrupt eukaryotic cells (Boman et al., 1991). This clearly implicates their possible usefulness as novel antibiotics. Although the exact mechanisms are not fully understood, cecropins have been shown to have direct action on bacterial membranes (Steiner et al., 1988). Determining the structure of the two cecropin homologues has revealed structural elements common among membrane-associated proteins (Steiner et al., 1988). Primary membrane attack is considered to be the cause of both Gram-positive and Gram-negative bacteria lysis (Steiner et al., 1988). Furthermore, binding sufficient amounts of cecropin to form a monolayer is necessary to modify the bacterial membranes.

It is believed that to provide protection against pneumonic pasteurellosis, *M. haemolytica* 1:A should not be allowed to colonize the URT. The antibacterial activity of cecropin B against *M. haemolytica* 1:A has not been reported previously. Cecropin B has

been shown to possess the broadest bactericidal activity of the cecropin family (Chen et al., 1997). It may be useful for eradication of pathogens from mucosal surfaces because it is highly effective against Gram-negative bacteria (Vaara et al., 1994). Cecropin B not only has the ability to effectively inhibit bacterial colonization, but it also neutralizes endotoxin. The very potent broad spectrum antibacterial activity of cecropin B makes it a likely candidate to prevent and/or treat infection. However, cost of production may limit this peptide's practical uses. The most cost-efficient peptide synthesis methods employed at the present time are still unreasonable (Hancock et al., 1998). One possible cost-effective approach would be to transfer the cecropin B gene into the species of interest and let the recipient's biosynthesis mechanisms synthesize the peptide. Although there are many indications that a transgenic or gene therapeutic approach may be useful in enhancing bacterial resistance in food animals, thereby reducing the use of conventional antibiotics, very few studies are available describing transfected mammals expressing cecropin B or cecropin B-like peptides.

CHAPTER 1

LITERATURE REVIEW

Classification, Economic Impact, and Habitat

The Bovine Respiratory Disease (BRD) Complex can be summarized as three major entities (Lillie, 1974). Clinically, these entities are known as enzootic pneumonia of calves, atypical interstitial pneumonia, and pneumonic pasteurellosis. Pneumonic pasteurellosis, commonly known as "shipping fever" was first described over a century ago (Mosier et al., 1989). Since then many aspects of the disease have been thoroughly investigated. Despite the extensive amount of research on pneumonic pasteurellosis, its continued prevalence in the feedlots is responsible for major economic losses in the North American beef cattle industry. More than a billion dollars are lost annually due to clinical disease in feedlot cattle (Brennan et al., 1998, Confer et al., 1994). Calves diagnosed with clinical pneumonic pasteurellosis experience increased weight loss during shipment, lower average daily gains, and poorer carcass quality. The disease is complex and multifactorial (Mosier et al., 1989). However one bacterium is most frequently associated with the disease (Biberstein et al., 1960). The major etiologic agent of bovine pneumonic pasteurellosis is *Mannheimia haemolytica* 1:A (Dalgleish, 1990, Frank, 1986). *M. haemolytica* 1:A (formally known as *Pasteurella haemolytica* 1:A) is a nonmotile, Gram-negative coccobacillus or rod, and a facultative anaerobe. *M. haemolytica* 1:A expresses many virulence factors which help to facilitate disease. These components include capsular polysaccharide, lipopolysaccharide (LPS) also known as endotoxin, and leukotoxin (Conlon et al., 1991, Whiteley et al., 1992). Other virulence factors may include outer membrane proteins (OMPs), a sialoglycoprotease, a

neuraminidase and possibly two types of fimbriae (Brennan et al., 1997, Lee et al., 1994, Whiteley et al., 1992).

M. haemolytica 1:A is considered to be part of the normal bacterial flora in the upper respiratory tract (URT) of healthy cattle, and can exist in the nasal cavity in very low numbers (Carter et al., 1991, Whiteley et al., 1992). It is believed that healthy cattle can carry undetectable levels of *M. haemolytica* 1:A for long periods of time (Frank, 1988). After shipment or other forms of stress, cattle may shed larger numbers of the bacterium (Frank et al., 1986). Furthermore, *M. haemolytica* 1:A is likely carried in one or more sites distinct from the nasal cavity, specifically the palatine tonsils (Frank et al., 1992). The tonsils provide an ideal site for carrying the bacterium due to their location (Frank et al., 1992, Lillie et al., 1974). The use a rifampicin-resistant strain of *M. haemolytica* 1:A allowed researchers to show that the organism colonized the tonsils and nasal cavity for more than two weeks in experimentally infected calves and commingled calves (Briggs et al., 1998). Tonsilar localization of *M. haemolytica* 1:A in healthy cattle allows for stress induced colonization of the nasal cavity and shedding of the organism.

The Normal and Transient Flora of the Bovine Nasal Passage

In addition to *M. haemolytica*, *Pasteurella multocida* and *Haemophilus somnus*, other bacteria commonly associated with BRD, the nasal passage of cattle is home to a diverse collection of bacteria that make up the normal and transient flora. Although a complete listing of all the bacteria found in the nasal passage of cattle does not appear to have been reviewed recently, the list of organisms cited in the literature is extensive. Some of the more frequently isolated bacteria from the URT include Gram-positive bacteria such as *Micrococcus, Corynebacterium, Staphylococcus, Streptococcus,* and

Bacillus as well as Gram-negative bacteria such as *Pseudomonas, Actinobacter, Moraxella, Neisseria catarrhalis*, and *Escherichia coli* (Corbeil et al., 1985, Woldehiwet et al., 1990). Other members of the bovine nasal flora may include Gram-positive bacteria such as *Lactobacillus, Streptomyces,* and *Diplococcus pneumoniae* as well as Gram-negative bacteria such as *Chromobacterium, Achromobacter, Serratia, Flavobacterium, Proteus,* and *Klebsiella* (Magwood et al., 1969). These species of bacteria have been found to start colonizing the URT as early as the calf's first day of life (Woldehiwet et al., 1990). The total bacterial load in the nasal passage increases with age, and is influenced by environmental temperature (Woldehiwet et al., 1990). Studies have shown that the population of bacteria in the nasal passage of calves fluctuates heavily in types and numbers (Magwood et al., 1969, Woldehiwet et al., 1990). Sometimes one genus or species is more dominant than others for random periods of time. As early as 1969 it was shown that *M. haemolytica* was capable of outnumbering the rest of the nasal flora for several days at a time (Magwood et al., 1969).

These bacteria, considered normal and transient nasal flora, may enhance or inhibit growth of *M. haemolytica*. *Bacillus* isolates were found to inhibit the growth of *M. haemolytica in vitro* (Corbeil et al., 1985). In contrast, several Gram-positive bacteria such as *Micrococcus, Corynebacterium,* and *Staphylococcus* were found to efficiently enhance the growth of *M. haemolytica in vitro* (Corbeil et al., 1985). The two Gram-negative bacteria *Moraxella* and *Actinobacter* enhanced *M. haemolytica* growth *in vitro* as well (Corbeil et al., 1985). Corbeil et al. showed that the normal and transient flora of the nasal passage can significantly affect the colonization of *M. haemolytica* 1:A.

Pathogenesis

Remaining in the URT of unstressed cattle, *M. haemolytica* 1:A causes no disease in the animal (Lillie, 1974). Under times of stress in the form of transportation, crowding, irregular feeding or watering, abrupt climate change, exposure to viral agents, or combination of the above, *M. haemolytica* 1:A proliferates in the URT (Frank et al., 1986, Yates, 1982). Stress also disrupts *M. haemolytica* 1:A localization, and allows the bacterium to colonize other parts of the respiratory tract. Conditions such as stress or cell damage and inflammation are ideal for *M. haemolytica* 1:A colonization and proliferation in the URT. Stressed cattle shed large numbers of *M. haemolytica* 1:A, which can be isolated during and immediately after shipment (Frank et al., 1986). Increased colonization and proliferation of *M. haemolytica* 1:A in the URT allows the organism to be inhaled into the lung (Frank, 1988). Researchers have shown that *M. haemolytica* 1:A can be isolated from droplet nuclei in tracheal air (Whiteley et al., 1992). In the lower respiratory tract (LRT), *M. haemolytica* 1:A would be easily cleared and no lung damage would occur under normal conditions (Shewen et al., 1982). However, chronic exposure to the lung allows *M. haemolytica* 1:A to overcome clearance mechanisms and to initiate pneumonia (Frank, 1988). Intranasal and intratracheal inoculation of *M. haemolytica* 1:A, as well as transthoracic inoculation of *M. haemolytica* 1:A directly into the lung have been shown to produce pneumonic lung lesions (Frank, 1986).

The respiratory tract immune system operates separately from the systemic immune system (McBride et al., 1996, McBride et al., 1999). In addition, the immune response in the upper URT differs significantly from the immune response in the LRT (Brennan et al., 1997). The immune response in the URT is specific, and not associated

with damage to the URT. While the immune response in the URT either prevents or controls *M. haemolytica* 1:A colonization, the immune response elicited by the organism in the LRT may cause the damage recognized clinically as fibrinous pleuropneumonia. The host immune response in the LRT is highly associated with acute pneumonic pasteurellosis (McBride et al., 1999). Chronic exposure to *M. haemolytica* 1:A in the lung induces inflammation, involving the influx of neutrophils and macrophages, extensive fibrin exudation, and enhanced vascular permeability (Whiteley et al., 1992, Yates, 1982). Inflammation and exudate as a result of the host immune response allows *M. haemolytica* 1:A to colonize the induced lesion by hindering clearance and providing an excellent iron-rich medium. *M. haemolytica* 1:A colonization in the lung and immune-mediated damage contribute heavily to the pathogenesis of pneumonic pasteurellosis. Histopathologic examinations of lung lesions reveal *M. haemolytica* 1:A and degrading inflammatory cells surrounding a foci of coagulation necrosis (Whiteley et al., 1992, Yates, 1982). Calves depleted of neutrophils have been shown to be somewhat resistant to lung lesion development (McBride et al., 1999). It is believed that neutrophils may be the primary effector of the disease. Neutrophils can be detected in lung lavage fluids within hours after *M. haemolytica* 1:A inoculation (McBride et al., 1999).

Leukotoxin and endotoxin secreted by *M. haemolytica* 1:A may also elicit an inflammatory response (Shewen et al., 1982). Using a neutrophil mediated pathway, leukotoxin damages primary endothelial cells, and kills neutrophils and alveolar macrophages (Frank et al., 1986, McBride et al., 1999). Killing neutrophils in the lung contributes to the early formation of lesions (Frank, 1986). The tissue destruction that

can lead to lesion formation is ultimately a result of the release of toxic oxygen radicals and proteases from neutrophils (Whiteley et al., 1991). Endotoxin directly interacts with the alveolar and pulmonary intravascular macrophages, causing them to synthesize proinflammatory cytokines TNFα, IL1, IL8, procoagulents, proteases and toxic oxygen radicals (Whiteley et al., 1991). It has been shown that endotoxin alone can initiate an inflammatory response in the lung (Whiteley et al., 1991).

One study suggests that immune complex disease (type III hypersensitivity) contributes to the development of the host inflammatory response (McBride et al., 1999), and that pneumonic pasteurellosis is in part an allergic pneumonitis. A Type III hypersensitivity to *M. haemolytica* 1:A antigens activates complement resulting in inflammation and neutrophilic influx. However, evidence suggests that *M. haemolytica* 1:A colonization in the lung and immune-mediated damage, including a type III hypersensitivity both play a role in the pathogenesis of bovine pneumonic pasteurellosis.

Vaccination Attempts

Vaccines which provide cattle with consistent immunity to pneumonic pasteurellosis are not yet available. Bacterins, combined bacterins, live, modified-live, viral, and component vaccines have been developed to protect cattle from shipping fever (Mosier et al., 1989). Most elicit some protection; however none of the bacterial immunogens have been able to consistently protect cattle in the field. *M. haemolytica* 1:A bacterins have been shown to be ineffective (Frank, 1986) and sometimes harmful. One belief is that the bacterins do not elicit antibody responses to the antigens important to pathogenesis (Frank, 1986). Researchers found that serum antibody titers correlated directly with resistance to lung challenge (Confer et al., 1983); however another

explanation for the failure of bacterins in the field is that they produce an IgG response (Frank, 1986, Mosier et al., 1989). Neutrophils phagocytize *M. haemolytica* 1:A opsonized by IgG, and in turn the neutrophils are killed by leukotoxin released by *M. haemolytica* 1:A (Frank, 1986). The result is immune-mediated lesion development.

Since viral infections were associated with *M. haemolytica* 1:A colonization in the lung, many viral vaccines have been developed (Mosier et al., 1989). Although vaccination of cattle against Parainfluenza 3 (PI_3) and Infectious Bovine Rhinotracheitis virus (IBRV) provided resistance to pneumonic pasteurellosis caused by *M. haemolytica* 1:A challenge under laboratory conditions (Frank, 1986), field trials of the vaccines were unable to provide cattle with protection (Mosier et al., 1989). Vaccination for Bovine Viral Diarrhea Virus (BVDV) was also targeted, however, as with the other viral vaccines failure in field experiments was attributed to the wide range of viruses that can elicit pneumonic pasteurellosis (Mosier et al., 1989). In addition cattle vaccinated with viral vaccines were still susceptible to *M. haemolytica* 1:A. In the next logical step, vaccines which combined *M. haemolytica* 1:A and viral antigens were developed. As with viral vaccines, combination vaccines provided cattle with protection under laboratory conditions, but failed in field experiments (Mosier et al., 1989). They also have the potential, like bacterins, to be more harmful.

Recombinant leukotoxin (rLKT) vaccines also met with little success (Conlon et al., 1991). Inoculating cattle with a rLKT vaccine did enhance the efficacy of another vaccine and decrease clinical signs as well as lung lesions; yet an immune response to leukotoxin alone did not protect cattle against pneumonic pasteurellosis (Conlon et al., 1991). Leukotoxin does play an important role in the pathogenesis of the disease, but

pneumonic pasteurellosis is multifactorial and complex. Other studies have shown that vaccination with various surface antigens such as LPS (endotoxin) and purified capsular polysaccharide also failed to protect cattle from pneumonic pasteurellosis (Confer et al., 1994).

Live and modified-live vaccines have been shown to be more effective in protecting cattle than any other type of vaccine (Mosier et al., 1989). Studies have shown live *M. haemolytica* 1:A vaccines to enhance resistance to experimental challenge (Confer et al., 1985). Although live and modified-live vaccines have been more effective at eliciting an immune response, they are still not consistent in field experiments. Most vaccines developed thus far have been able to produce some immune response. However, previously developed vaccines all have failed to provide cattle with consistent resistance to *M. haemolytica* 1:A and pneumonic pasteurellosis in the field.

Antibiotic Therapy

The use of antibiotics to treat pneumonic pasteurellosis has been generally successful (Frank, 1986). Antibiotics are chemical substances of microbial origin. They can either destroy or inhibit the growth of bacteria. Antibiotics such as tetracyclines and sulfonamides have a broad spectrum of activity against a wide range of bacteria, while antibiotics such as fluoroquinolones have a narrow spectrum of activity. Antibiotics have four mechanisms of action. They may inhibit cell wall synthesis, cell wall function, nucleic acid synthesis, or protein synthesis. In the past, antibiotics were purified from media filtrates. Later they were synthesized. Today new antibiotic drugs are biosynthesized from chemical modification of molecules.

Most veterinarians rely on antimicrobial agents to treat pneumonic pasteurellosis. Antimicrobial therapy is a very effective method for both the treatment and prevention of BRD (Watts et al., 1994). The veterinarian may select antibiotics for treatment on the basis of perceived efficacy, cost, convenience, availability, toxicity, and residue profile (Watts et al., 1994). Veterinary diagnostic labs assist the veterinarian by performing susceptibility tests and providing consistent interpretations of the results (Shyrock et al., 1996). By monitoring the susceptibility trends of *M. haemolytica* 1:A, veterinarians can select the most effective antibiotics. With the occurrence of antibiotic resistant strains of bacteria, the appropriate use of antibiotics should include exposing *M. haemolytica* 1:A to sufficient concentrations of the antimicrobial agents for an appropriate duration as to minimize the development of resistant strains (Watts et al., 1994).

β-lactam antibiotics such as ampicillin, which inhibit bacterial cell wall synthesis have been used to treat pneumonic pasteurellosis in the past. However, ampicillin resistant strains of *M. haemolytica* 1:A have been frequently encountered (Hartman et al., 1993, Watts et al., 1994). A relatively new β-lactam antibiotic, ceftiofur, which is a cephalosporin with an extended spectrum of activity, was shown to be very effective against *M. haemolytica* 1:A (Watts et al., 1994). Using a *M. haemolytica* 1:A challenge model researchers were able to show that a single subcutaneous injection of ceftiofur crystalline-free acid sterile oil suspension (CCFA-SS, 100 mg ceftiofur equivalents (CE)/ml) of 4.4 to 8.8 CE/kg of body weight was very effective at inhibiting colonization in the lung (Hibbard et al., 2002). The formulation was long-acting and more effective than tilmicosin, though alternative sites for injection must be found because subcutaneous injections in the neck left ceftiofur residues for extended periods of time (Hibbard et al.,

2002). In a four year survey to monitor the emergence of antibiotic resistant strains of *M. haemolytica* 1:A no strains were found to be resistant to ceftiofur (Watts et al., 1994).

Antibiotics which inhibit bacterial nucleic acid synthesis such as sulfonamides and sulfonamide-trimethoprim combinations have been used to treat pneumonic pasteurellosis in the past as well (Watts et al., 1994). Studies have shown *M. haemolytica* 1:A to have an increasing pattern of resistance to these antibiotics (Hartman et al., 1993). It has been speculated that resistance to sulfamethazine is so widespread that its usefulness in cattle is limited (Watts et al., 1994). Flouroquinolones including ciprofloxacin, norfloxacin, and enrofloxacin are entirely man made antibiotics, which have remarkable potency against many aerobic and facultative anaerobic bacteria (Prescott et al., 1990). All three antibiotics were shown to have a minimum inhibitory concentration (MIC) of less than or equal to 0.06 µg/ml for 100% of the *M. haemolytica* 1:A isolates tested *in vitro* (Prescott et al., 1990). Enrofloxacin, which is approved for use in cattle, was shown to have a 0.03 µg/ml MIC for 100% of the *M. haemolytica* 1:A isolates tested (Prescott et al., 1990).

Streptomycin, tetracyclines, spectinomycin, chloramphenicol, erythromycin, florfenicol, and tilmicosin all inhibit bacterial protein synthesis. Streptomycin, tetracyclines, spectinomycin, chloramphenicol and erythromycin resistant strains of *M. haemolytica* 1:A have been frequently encountered (Hartman et al., 1993, Watts et al., 1994). In a four year survey to monitor the emergence of antibiotic resistant strains of *M. haemolytica* 1:A, tetracycline and spectinomycin were shown to have MICs of 32 µg/ml and 64 µg/ml respectively to 90% of the isolates tested (Watts et al., 1994).

Administering florfenicol to cattle upon arrival at a stockyard was shown to decrease the detection of *M. haemolytica* 1:A in the nasopharynx (Frank et al., 2002). Tilmicosin is a relatively new macrolide antibiotic, with mostly anti-Gram-positive bacterial activity. However, this macrolide can inhibit some Gram-negative organisms including *Mannheimia*, *Pasteurella*, *Haemophilus* and *Actinobacillus* bacteria (Shyrock et al., 1996). *In vitro* studies have shown tilmicosin to have significant antibacterial activity against *M. haemolytica* 1:A (Hartman et al., 1993). Tilmicosin was shown to have an MIC of less than or equal to 2 μg/ml for 96% of the *M. haemolytica* 1:A isolates tested (Hartman et al., 1993). However, in the four year survey to monitor the emergence of antibiotic resistant strains of *M. haemolytica* 1:A, tilmicosin effectiveness was found to vary substantially (Watts et al., 1994). Due to increased resistance to erythromycin, another macrolide, the usefulness of tilmicosin in the treatment of and protection from pneumonic pasteurellosis may decrease (Watts et al., 1994).

Public concern for the use of antibiotics in food animals is once again gaining momentum. Although the use of antibiotics may result in significantly harmful residue levels in food meant for human consumption, these illegal drug residues are no longer being found in carcasses. The U.S. Department of Agriculture's (USDA) Food Safety and Inspection Service (FSIS) ensure that antibiotic residues in food products are within established limits. The concern has now shifted to drug-resistant microbes. Many producers use low doses of antibiotics as growth promoters (Ferber, 2003). The result is an increased occurrence of antibiotic resistant bacteria, which may pose a threat to human health (Ferber, 2003). An effort to eliminate the routine use of antibiotics in food animal production is underway (Ferber, 2003). In 1996, the U.S Food and Drug Administration

(FDA), U.S. Centers for Disease Control and Prevention (CDC), and USDA began coordinating under the National Antimicrobial Resistance Monitoring System's (NARMS) Enteric Bacteria program in an effort to provide objective information and analysis about trends of antibiotic resistance in animal bacteria due to agricultural practices.

Preventing Colonization of the Nasal Passage

The immune response in the URT differs significantly from the immune response in the LRT (Brennan et al., 1997). While the immune response in the URT either prevents or controls *M. haemolytica* 1:A colonization, the immune response elicited in the LRT may cause damage. The immunoglobulin A (IgA) comprises 90% of the antibody present in the URT (McBride et al., 1996). This is much greater than in the LRT. In one study, IgA was not detected at all in lung lavage fluids of cattle receiving intrapulmonary inoculations of *M. haemolytica* 1:A (McBride et al., 1999). Although IgA is present in the LRT, IgG_1 is the most dominant antibody in the lung (Brennan et al., 1997). Both IgG_1 and IgA have been associated with enhanced *M. haemolytica* 1:A clearance in the lung (McBride et al., 1996). However both immunoglobulins have also been implicated in contributing to the inflammatory response leading to acute lung lesions (Frank, 1986, Johnson et al., 1986).

In the nasopharynx, IgA and a portion of the IgG_1 are locally produced (Duncan et al., 1972). By reducing the ability of bacteria to invade mucosal surfaces, IgA is believed to be the first line of defense against *M. haemolytica* 1:A colonization in the URT (Roth, 1991). Since critical numbers of *M. haemolytica* 1:A must be aspirated into the lung to cause pneumonic pasteurellosis (Frank, 1988), reducing the levels of *M.*

haemolytica 1:A colonization in the nasopharynx would provide cattle with protection against the disease. If IgA antibodies could reduce *M. haemolytica* 1:A colonization in the nasopharynx, then finding ways to induce a secretory IgA response on the mucosal surfaces in the nasal cavity are paramount. When attempting to induce mucosal immunity, the route of vaccine administration is important (Roth, 1991). Administering the vaccine intranasally is beneficial when trying to induce a secretory IgA response. Studies have shown that intranasal vaccination can play a key role in inducing an immune response to *M. haemolytica* 1:A in the nasopharynx (Brennan et al., 1998). Aerosol vaccination at the level of the middle meatus in the nasal cavity of cattle was shown to enhance *M. haemolytica* 1:A clearance from the nasopharynx and prevented migration of *M. haemolytica* 1:A into the lung (Brennan et al., 1998). Immunoglobulin A antibodies were detected in the nasal secretions of these cattle. This evidence supports a previous study in which researchers trying to colonize the nasal passages of cattle found that after rapid clearance of *M. haemolytica* 1:A, the calves responded with antibody titers detectable in nasal secretions (Frank, 1988). However the use of currently available vaccines does not allow enough time for an effective immune response to develop before the onset of pneumonic pasteurellosis in calves at the feedlots (Frank et al., 2002).

It appears there have been very few studies aimed at the use of antibiotics to eliminate colonization of the nasal passage. The use of an effective antibiotic to inhibit the rapid colonization of *M. haemolytica* 1:A in the nasopharynx should reduce the occurrence of pneumonic pasteurellosis in cattle arriving at the feedlots. The use of oxytetracycline or the feeding chlortetracycline at the feedlots did not reduce or eliminate the colonization of *M. haemolytica* 1:A in the nasopharynx (Frank et al., 2000b). In

calves experimentally infected with *M. haemolytica* 1:A, tilmicosin was effective in alleviating clinical signs and pathology findings of pneumonic pasteurellosis as well as the presence of viable organisms in the lung (Morck et al., 1997). Furthermore, a single subcutaneous injection of 10mg per kg body weight protected the lung for up to 72 hours with concentrations well above the MIC for *M. haemolytica* 1:A (Morck et al., 1997). Researchers have also shown that a single dose of 10 mg per kg body weight of tilmicosin was more effective at reducing mortality due to pneumonic pasteurellosis than ceftiofur, oxytetracycline, or trimethoprim-sulfadoxine (Shyrock et al., 1996). In a study focused on decreasing *M. haemolytica* 1:A colonization in the nasopharynx, both florfenicol and tilmicosin were shown to be effective (Frank et al., 2000a, Frank et al., 2002). Florfenicol inhibited *M. haemolytica* 1:A colonization in the nasal passage for up to 4 days, while tilmicosin inhibited *M. haemolytica* 1:A colonization in the nasal passage for up to 6 days (Frank et al., 2002). There appears to be no advantage to using tilmicosin before or after arrival at the stockyard (Frank et al., 2000b). Using an antibiotic such as tilmicosin before or after transport can reduce the occurrence of pneumonic pasteurellosis at the stockyards, and would also allow vaccines given before transport time to elicit an immune response (Frank et al., 2000b).

Clinical pneumonic pasteurellosis seems inevitable only after *M. haemolytica* 1:A colonization and proliferation occurs in the URT. Preventing *M. haemolytica* 1:A from ever colonizing the nasopharynx of cattle would certainly be ideal, although unrealistic. Simply controlling the prolific growth of *M. haemolytica* 1:A by antimicrobial treatment or by eliciting an antibody response in the nasal passage would reduce the number of

organisms reaching the lungs. Reduced numbers of bacteria in the LRT would be easily cleared with no lung damage, thereby preventing pneumonia before it is started.

Insect Immunity

Insects, like vertebrates, respond to infection with a highly effective immune system, both cellular and humoral (Boman et al., 1991). Although lymphocytes have never been found in insects, and they were once thought not to possess any form of immunoglobulins, these invertebrates still have a specialized immune system (Boman et al., 1987). However, insect immunity still lacks the high degree of specificity found in the immune systems of vertebrates (Boman et al., 1987). An injury is often the cause of infections, for this reason insects will often respond to an injury and other forms of infection in a similar manner (Boman et al., 1991).

In regards to cellular immunity, different hemolymph cell types have important roles in protecting insects. Generally, six cell types are recognized (Boman et al., 1987). They include prohemocytes, plasmatocytes, granulocytes, coagulocytes, spherulocytes and oenocytes. Plasmatocytes and granulocytes, which are granular cells, phagocytize smaller bacteria and unicellular fungi (Boman et al., 1987). Many of the hemocytes work in cooperation to encapsulate large aggregates of invading cells or parasites such as nematodes (Boman et al., 1987). Insects respond to large numbers of invading cells by the formation of nodules (Boman et al., 1987). In addition to being involved in phagocytosis, plasmatocytes are also involved in capsule and nodule formation (Boman et al., 1987).

Insects respond to an injection of live bacteria by rapidly removing them from the hemolymph with phagocytosis and nodule formation (Boman et al., 1987). The rapid

clearance of bacteria is followed by a short period of RNA synthesis and then subsequent protein synthesis (Boman et al., 1991). The insect is then considered to be immunized due to the increased circulation and activity of antibacterial agents in the hemolymph (Boman et al., 1987). In all, approximately 15 to 20 immune proteins are synthesized in the humoral immune response. In diapausing pupae of the saturniid *Hyalophora cecropia*, also known as the giant silk moth, this increase in antibacterial activity is mainly due to the synthesis of three novel classes of antibacterial proteins, specifically lysozyme, attacins and cecropins (Boman et al., 1991). These immune factors were first purified in 1980, and were first discovered in *H. cecropia*.

Lysozyme was the first antibacterial protein purified from insects (Boman et al., 1987). Since its initial discovery, lysozyme has been found in many other insects (Boman et al., 1991). Lysozyme is an enzyme, therefore it has catalytic activity. It was found to contain 120 amino acid residues, a substrate binding groove and four disulfide bonds (Boman et al., 1987). Lysozyme can be isolated from the larvae of nonimmunized *H. cecropia*, but not in the pupae. However, lysozyme can be induced in both by the injection of nonpathogenic bacteria (Boman et al., 1987). It is not the main antibacterial protein responsible for immunity in insects because insects can still eliminate many lysozyme-resistant bacteria (Boman et al., 1991). It is believed that lysozyme works in concert with attacins and cecropins in *H. cecropia*, and probably in concert with the main antibacterial proteins in other insects. Lysozyme has been shown to have limited antibacterial activity. It is only bactericidal to a few Gram-negative bacteria such as *Bacillus megaterium, B. subtilis,* and *Micrococcus luteus* (Boman et al., 1991). Cecropins A and B have also been shown to kill *B. megaterium* and *M. luteus* (Boman et

al., 1991). *B. subtilis* is the only organism shown to be sensitive to lysozyme but resistant to cecropins. The main function of lysozyme may not be bactericidal, but to clear the murein sacculus or peptiglycan and cell wall material left after the action of attacins and cecropins (Boman et al., 1987).

Attacins were first isolated from the immune hemolymph of *H. cecropia* pupae injected with live bacteria (Boman et al., 1987). Six different attacins were isolated and found to contain 183 to 188 amino acid residues (Boman et al., 1991). Furthermore, it was found that attacins existed in two forms, basic and acidic peptides. It is believed that attacins work in concert by facilitating the action of lysozyme and cecropins (Boman et al., 1991). It was shown that attacins only act on growing bacteria, and that their antibacterial activity is limited to a small number of bacterial species (Boman et al., 1991). In larvae of the Chinese Oak Silk Moth, *Antheraea pernyi*, attacin was found to inhibit *Escherichia coli* and two other bacteria in the gut (Boman et al., 1987). It was shown that the bactericidal action of attacins is directed to the outer membrane (Boman et al., 1991).

Of the three families of antibacterial proteins in *H. cecropia*, the cecropins have the most potent bactericidal activity (Boman et al., 1987, Chen et al., 1997). The cecropins were discovered in 1980 when Hans Boman and his research group successfully separated them biochemically and functionally from lysozyme in *H. cecropia* (Boman et al., 1991). Cecropins A and B were isolated initially (Boman et al., 1987), since then this family has been extended to include cecropins C, D, E, and F (Chen et al., 1997, Hultmark et al., 1982). The cecropins have highly homologous sequences consisting of 35 to 39 amino acid residues. Cecropins in general have a broad spectrum

of antibacterial activity against several Gram-positive and Gram-negative bacteria (Boman, 2003). There appears to be little known about the minor cecropins C, E, and F; cecropins A and B have been shown to be highly active against both Gram-positive and Gram-negative bacteria, while cecropin D has shown high activity only against *E. coli* and *Acinetobacter calcoaceticus* (Boman et al., 1991). Cecropin B is slightly more potent than cecropin A. Furthermore, cecropins have been shown to lyse bacteria, while leaving eukaryotic cells intact; unless they have been transformed (Boman et al., 1991, Moore et al., 1995). Since they were originally found in *H. cecropia*, cecropins have been found in other lepidopterans (butterflies and moths), dipterans (flies, etc.), other insect orders, pigs, and a marine protochordate (Hancock et al., 1998).

It was once thought that all invertebrates including insects did not possess any form of immunoglobulins. Hemolin was the first immunoglobulin-like molecule thought to be part of the immune response, isolated from an invertebrate (Boman et al., 1991). Like lysozyme, attacins, and cecropins, hemolin was first characterized from the hemolymph of *H. cecropia* (Boman et al., 1991). Although little is known about hemolin, it is the most abundant immune protein in *H. cecropia*. It has no antibacterial activity, but has been shown to bind bacteria *in vitro* (Boman et al., 1991). Once bound, hemolin complexes with another hemolymph protein. This complex is then bound by hemocytes, causing it to disaggregate. Studies indicate that hemolin is involved in self/nonself recognition (Boman et al., 1991). Hemolin is produced as part of an immune response, but can preexist in hemolymph and the fat body (Boman et al., 1991).

Insect orders other than lepidoptera produce lysozyme, attacin-like, and cecropin-like peptides as well as unique antibacterial peptides of their own. The fly, *Phormia*

terranovae, produces diptericin in response to infection (Boman et al., 1991). Honeybees produce apidaecins and abaecin (Boman et al., 1991). The main component of bee venom, melittin, which has some bactericidal activity, has been shown to be similar in structure to cecropin (Boman et al., 1991).

The Cecropin Gene

The cecropin locus in *H. cecropia* is about 20 kb long (Boman et al., 1991). The transcriptional region of cecropin B was the first to be sequenced from *H. cecropia* (GenBank Accession No. X07404) (Boman, 2003, Xanthopoulos, et al., 1988). The sequence was shown to include a 0.79 kb *Bg*/II fragment and an adjacent 0.53 kb *Bg*/II-*Pvu* I fragment (Xanthopoulos et al., 1988). The gene is a typical eukaryotic gene. A consensus sequence, known as the TATA box, is found in the promoter region of most genes transcribed by eukaryotic RNA polymerase II. The consensus sequence is generally 5'-TATAAAA-3', and is important in determining the accurate position at which transcription is initiated. The cecropin B gene contains a 5'-TATAAA-3' sequence at position –30 in the 5' flanking region, which is homologous to the TATA box consensus (Fig. 1.1) (Boman, 2003, Xanthopoulos et al., 1988). Most eukaryotic genes also have another consensus sequence in the promoter located upstream of the start of transcription. This sequence, 5'-GG T/C AATCT-3', is a requirement for transcription to proceed smoothly. In the gene coding for cecropin B the CAT box is present as the sequence 5'-CAAAT-3' found at position –68 (Fig. 1.1) (Boman, 2003, Xanthopoulos et al., 1988). Also a polyadenylation signal represented by the sequence 5'-AATAAA-3' or 5'-ATTAAA-3' is required for the transcription of eukaryotic genes. The cecropin B gene was shown to have two such signals of the sequence 5'-AATAAA-3', located in the

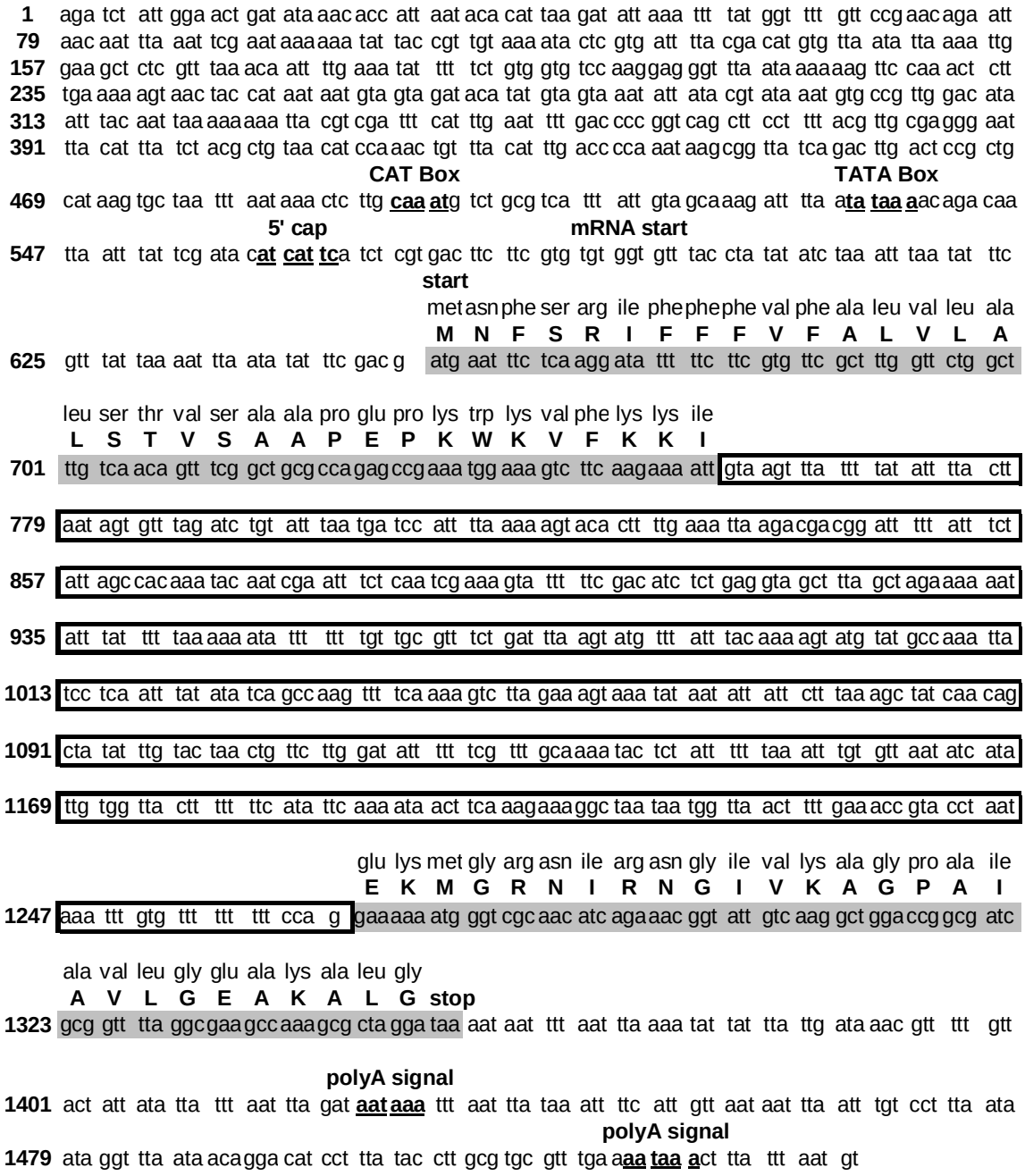


Figure 1.1. The cecropin B gene **=exon** **=intron**

3’ end of the gene (Fig. 1.1) (Boman, 2003, Xanthopoulos et al., 1988). The gene also contains a conserved, insect specific 5’ cap site sequence, 5’-ATCATTC-3’ (Fig. 1.1) (Boman, 2003, Xanthopoulos et al., 1988).

Cecropin B is synthesized as a prepro molecule, and there is one transcriptional unit for preprocecropin B. The coding region contains two exons separated by only one intron (Fig. 1.1). The transcriptional unit is 1035 bp long from the cap site to the second polyadenylation signal. The gene contains a 90 bp-long mRNA untranslated region prior to the start codon (AUG) (Xanthopoulos et al., 1988). The signal peptide, the prosequence, and the first eight amino acid residues of mature cecropin B peptide are translated from the first exon. A single 514 bp intron separates the first exon from the second exon. The second exon contains the rest of the code for mature cecropin B plus one glycine residue (Fig. 1.1) (Xanthopoulos et al., 1988).

Xanthopoulos et al. suggested that there may be 3 to 5 copies of the gene for cecropin B present in the *H. cecropia* genome. Since cecropins A, B, and D show 62% to 65% homology, they most likely originated from gene duplications. Furthermore, it has been shown that transcripts for cecropin A and B are made simultaneously while there is a slight delay for the transcript of cecropin D (Boman et al., 1991). Kinetic activities of these three cecropins have shown a similar delay in cecropin D antibacterial activity (Boman et al., 1991).

Certain hemocytes are only slightly responsible for the synthesis of immune proteins in *H. cecropia* (Boman et al., 1991). A specialized organ known as the fat body is believed to be the main organ responsible for the synthesis of immune proteins in both larvae and pupae of *H. cecropia*. *In vitro* studies have shown that fat bodies dissected

from immunized *H. cecropia* pupae could synthesize active immune proteins (Boman et al., 1991). It was further shown that LPS was enough to induce isolated fat bodies to produce immune proteins (Boman et al., 1991).

Cecropin B Structure and Activity

The cecropins are strongly basic peptides containing 35 to 39 amino acid residues. The NH_2-terminal region is strongly cationic, while the COOH-terminal region is a long hydrophobic stretch (Boman et al., 1991). All cecropins have a highly conserved tryptophan at position 2, single and double lysines at positions 5, 8, and 9, and arginine at position 12 (Fig. 1.2). Due to these conserved sequences found in several different species of insects there must have been strong selection pressure for the cecropin genes (Boman et al., 1991).

Lys(K)-Trp(W)-Lys(K)-Val(V)-Phe(F)-Lys(K)-Lys(K)-Ile(I)-Glu(E)-Lys(K)-Met(M)-Gly(G)-Arg(R)-Asn(N)-Ile(I)-Arg(R)-Asn(N)-Gly(G)-Ile(I)-Val(V)-Lys(K)-Ala(A)-Gly(G)-Pro(P)-Ala(A)-Ile(I)-Ala(A)-Val(V)-Leu(L)-Gly(G)-Glu(Ala(A)-Lys(K)-Ala(A)-Leu(L)-NH2

Figure 1.2. Primary structure of cecropin B

All cecropins are synthesized as larger prepro molecules with about 62 to 64 amino acid residues (Boman et al., 1991). In the NH_2-terminus about 24 to 26 of the amino acid residues are not present in any mature cecropin protein. About 22 of them are probably part of the signal peptide (Boman et al., 1991). The amino acid sequence of preproceropin B suggests that the primary translation product is processed in two, maybe three steps to yield mature cecropin B (Van Hofsten et al., 1985). Cecropin B is translated as a 62 amino acid residue precursor molecule including a leader peptide and a COOH-terminal glycine residue (Fig. 1.1). The leader or signal peptide is 26 amino acid residues long on the NH_2-terminus. *In vitro* studies have shown that the leader peptide

could be removed by one or more protolytic enzymes (Van Hofsten et al., 1985). Following the activity of membrane-bound signal peptidase, a dipeptidypeptidase could recognize the two dipeptide units in Ala-Pro-Glu-Pro (Fig. 1.1) that precede the NH_2-terminus of mature cecropin B (Van Hofsten et al., 1985). Natural cecropin B was found to have an amide group bound to the COOH-terminal leucine residue (Fig. 1.2) (Van Hofsten et al., 1985). It is likely that another step in the processing of preprocecropin B involves the donation of nitrogen from the COOH-terminal glycine residue found in the precursor molecule (Fig 1.1).

All the endogenous antimicrobial peptides found in plants and animals have common structural motifs. They are typically cationic, containing excess lysine and arginine residues, amphipathic molecules composed of 12 to 45 amino acid residues (Hancock et al.,1998). Some are α-helical structures, especially in anionic solvents or mixed with anionic phospholipid membranes. Others contain β-sheet secondary structures stabilized by intramolecular cysteine disulfide bonds.

The structure of cecropin B as predicted by sequence analysis as well as circular dichroism spectroscopy, and determined by Nuclear Magnetic Resonance (NMR) of cecropin A is a near-perfect NH_2-terminal amphipathic α-helix, and a COOH-terminal hydrophobic α-helix joined by a flexible hinge at positions 23 and 24 (Fig. 1.2, Gly and Pro) (Chen et al., 1997, Durell et al., 1992, Moore et al., 1995). The amphipathic α-helix contains both a hydrophilic and a hydrophobic (lipophilic) group. The helix-bend-helix structural motif is common among many cationic lytic peptides (Hancock et al., 1998). The NH_2-terminus of cecropin B is highly basic due to a number of lysine (n=6) and arginine (n=2) residues. An unusually polarized NH_2-terminal amphipathic helix is also a

very common structural motif of membrane associated proteins (Steiner et al., 1988). Due to a lysine residue and amidation on the end, the hydrophobic COOH-terminal α-helix also has a positive charge. Cecropin B, as well as all cecropins, is devoid of cysteine residues (Moore et al., 1996).

The lytic activity of cecropin has been widely studied. Cecropin B can cause rapid lysis of bacterial cell membranes (Boman, 2003, Steiner et al., 1998). There are two theories explaining the action of cecropin B. The most likely mechanism of action involves aggregation and insertion of the peptides into the membrane, pore formation, and then subsequent membrane disruption (Hancock et al., 1998, Wang et al., 1999). The alternative hypothesis states that the peptides may accumulate on the membrane causing cooperative permeabilization (Hancock et al., 1998). This destabilization of the membrane has been dubbed the 'carpet effect' and is comparable to the activity of a detergent (Wang et al., 1999). Evidence has shown that the rate of lysis is dependent on the concentration of cecropin (Boman et al., 1991). Binding sufficient amounts of cecropin to form a monolayer is necessary to modify the bacterial membranes (Steiner et al., 1988). Bacteria which are resistant to cecropin still bind large amounts in a non-productive manner. Eukaryotic cells are resistant because they avoid the binding of cecropin B. The indication is that cecropins act stoichiometrically. The activity of cecropin B is a matter of mass action, and is not catalytic in nature (Steiner et al., 1988).

In vitro and *in vivo* studies have shown that cecropins possess both antibacterial and anticancer activity (Moore et al., 1995, Moore et al., 1996). Cecropins are thought to induce ion channels in membranes followed by cell lysis. The rapid membrane disruption of bacterial cells induced by cecropin B is most likely caused by primary

membrane attack (Steiner et al., 1988). By assimilating knowledge of cecropin and other antibacterial peptides into three dimensional atomic-scale computer models researchers predicted that cecropins may assemble in membranes to form two types of ion channels (Durell et al., 1992). Type I ion channels may be formed by a collection of bound dimers with the pore formed by the transmembrane COOH-terminal helices (Durell et al., 1992). These dimers could then be arranged into a hexagonal lattice. This could explain the large amounts of cecropin that bind to the bacterial membrane (Steiner et al., 1988). If a concerted conformational change of a Type I ion channel in which the pore was formed by the NH_2-terminal helices would occur, a larger Type II ion channel would be formed (Durell et al., 1992). Cecropins as well as other antimicrobial peptides have been shown to form ion channels on artificial membranes such as liposomes (Boman et al., 1991). Cecropin B has been shown to lyse negative and neutral liposomes, but not positive ones (Steiner et al., 1988, Vaara et al., 1994). It is believed that negatively charged headgroups on the membrane surface attract and bind the positively charged NH_2-terminal helices (Durell et al., 1992). The hydrophobic COOH-terminal helices are then inserted into the membrane core. The positively charged NH_2-terminal helices are pushed into the membrane by positive potential on the same side of the membrane as the peptides (Durell et al., 1992). Transmembrane NH_2-terminal helices become associated with the hydrophilic residues faced inward forming an aqueous pore; the hydrophobic residues then are in contact with the aliphatic phase of the membrane.

Changing the structure of the NH_2-terminal α-helix of cecropin B lowers its membrane binding affinity (Steiner et al., 1988). An antibacterial peptide containing two amphipathic α-helical segments derived from cecropin B was shown to have a similar

ability to lyse bacteria with a 2 to 6 fold higher potency in lysing cancer cells when compared to cecropin A (Srisailam et al., 2000). The researchers summarized that an amphipathic α-helix is indeed necessary for performing effective cell killing activity. Furthermore, it was recently shown that cecropin B and cecropin B analogues which contain at least one amphipathic α-helix lyse cells by the formation of pores (Chen et al., 2003). In contrast, B analogues which lack an amphipathic α-helix appear to follow the 'carpet effect' model.

Lytic peptides, which were designed to have extra cationic residues, were less effective in breaking liposomes and killing bacteria but more effective in lysing cancer cells (Chen et al., 1997). It is possible that the content of the α-helix is not the main factor of antibacterial and anticancer activity, but that the characteristics of the α-helix itself may be important. When eukaryotic cells are transformed they usually have more exposed anionic lipids, and microvilli on the cell membrane tend to make the surface more accessible (Chen et al., 1997). The greater susceptibility to cationic lytic peptides of cancer cell membranes may be a possible explanation for the difference between the lytic activities on eukaryotic cells versus eukaryotic cancer cells.

In another study, researchers tested the effect of pH on the ability of cecropin B to lyse liposomes of low or high levels of anionic content. They were able to show that at high pH, cecropin B reached maximum lytic activity on both types of liposomes (Wang et al., 1999). Here, the researchers summarized that pH is a major factor in the ability of cecropin B to effectively lyse membranes and that secondary structure and binding ability may not be the main determinants of lytic peptide activity.

Although cecropins have a broad spectrum of activity against both gram-positive and Gram-negative bacteria, the latter are generally more susceptible to cecropins (Boman, 2003, Moore et al., 1996). The cytoplasmic membrane is the predicted site of the lethal action of cecropin B. The cytoplasmic membrane of Gram-positive bacteria is inherently more resistant to cecropin B (Boman, 2003, Moore et al., 1996). In Gram-negative bacteria, cecropin B must be able to cross the outer membrane to access the cytoplasmic membrane. There is evidence to suggest that cecropin B disrupts the bacterial outer membrane. After treatment with cecropin B, electron microscopy can be used to show the outer membrane disruption of *E. coli* (Moore et al., 1996). If the peptide initially binds through electrostatic attraction, this could explain the efficacy of cecropin B on the outer membranes of Gram-negative bacteria. This electrostatic attraction may be absent or reduced in the less susceptible Gram-positive bacteria. Cecropin B initially interacts with the highly anionic outer membrane glycolipid, LPS, of Gram-negative organisms (Hancock et al., 1998). Then the peptide accesses the cytoplasmic membrane by local disruption of the outer membrane. Consequently, a highly anionic outer membrane may not be completely necessary. One study showed that cecropin B was almost as active on wild-type enteric bacteria as it was on their mutant strains that have very defective outer membranes (Vaara et al., 1994). Furthermore, mutant strains which had less anionic LPS were completely susceptible to cecropin B (Vaara et al., 1994). Even though the natural L-isomer and the D-enantiomer of cecropin B both form α-helical secondary structures (inverse conformation), they differ in their interaction with the chiral acidic sites of LPS (Bland et al., 2001). When compared to L-

cecropin B however, the difference in LPS interaction does not affect D-cecropin B antibacterial activity against Gram-negative bacteria.

In summary, cecropin B is not only one of the most potent broad spectrum lytic peptides, but it is also very effective against Gram-negative organisms. It can cause rapid, even instantaneous lysis, of bacteria cells by disrupting the cytoplasmic membrane. The strong cationic amphipathic α-helix is required for effective binding to the bacterial membrane, but does not function through chiral-specific receptors. Primary membrane attack through the formation of ion channels is the most likely cause of cell lysis. However, the density of bound cecropin B must be high for such pores to form.

Cecropins in the Animal Kingdom

Cecropin homologues have been isolated from *Antheraea* pupae, the flesh-fly *Sarcophaga*, the silkworm *Bombyx,* the tobacco hornworm *Manduca, Drosophila,* the blood cells of a marine protochordate, and even porcine intestines (Boman et al., 1991, Hancock et al., 1998, Marshall and Arenas, 2003). These findings suggest that cecropins may be widespread throughout the animal kingdom. In every species they contribute to the animal's innate immunity.

Cecropin P_1 was discovered when researchers isolated anti-*E. coli* factor from the upper part of the porcine small intestine (Lee et al., 1989). Sequence analysis determined this factor to be a 31 amino acid residue, cysteine-free, cecropin-like peptide. The secondary structure is not the helix-bend-helix motif found in cecropin B, but a single long α-helical structure (Srisailam et al., 2000). The NH_2-terminus begins with an amphipathic structure of 4 to 5 turns followed by a shorter hydrophobic region of 1 to 2 turns. Also, the carboxyl group on the COOH-terminus of natural cecropin P_1 is not

amidated (Lee et al., 1989). It has been shown that cecropin P_1 kills bacteria by instantaneous lysis, and more peptide is required to kill more bacteria (Boman et al., 1993).

Other Antimicrobial Peptides

Cecropins were first isolated from insects, then subsequently from mammals (Boman et al., 1987). This contradicted the belief that the use of antimicrobial peptides, especially cecropins, was limited to the insect immune response. Soon after defensins were first discovered in mammals, they were subsequently found in insects (Lee et al., 1989). These findings suggested that not only cecropins, but all antimicrobial peptides are widely distributed in the animal kingdom. This distribution is most likely due to the fact that RNA and protein synthesis of antimicrobial peptides is much faster and easier than facilitation of the mechanisms dependent also on cell proliferation (Lee et al., 1989). Hence antimicrobial peptides provide fast and easy defense. In addition to diptericin from flies and apidaecins and abaecin from honeybees, researchers have found lytic peptides in other insects, bacteria, crustaceans, amphibians, and mammals. (Boman et al., 1991, Hancock et al., 1998, Marshall and Arenas, 2003, Moore et al., 1996). These peptides include defensins, magainins, bactenecins, lactoferricins, and cathelicidins as well as tachyplesin, protegrin, indolicidin, and nisin. The innate immunity of most animals relies heavily on the protection provided by antimicrobial peptides.

To protect their skin against microorganisms, frogs produce the antimicrobial peptides known as magainins (Boman et al., 1991). Magainins were first isolated from the skin of the African clawed frog, *Xenopus laevis* (Hancock et al., 1998). Magainins are 23 amino acid residues long, lysine-rich, cysteine-free, and are believed to form α-

helical secondary structures. They are synthesized in the granular gland which stores large amounts of active peptides. Magainins are active against many Gram-positive and Gram-negative bacteria. They have been shown to form pores in the cell membrane, but are relatively nontoxic to untransformed eukaryotic cells (Hancock et al., 1998).

Defensins were first discovered in mammals then subsequently in insects (Lee et al., 1989). Cecropins and defensins are very different types of molecules. Defensins are cysteine-rich and form β-sheet secondary structures as opposed to cysteine-free α-helical secondary structures (Hancock et al., 1998). Due to unique consensus sequences and placement of disulfide bonds they have been divided into two families, classical and β-defensins (Selsted et al., 1996). All defensins are similar in that they have identical folds and six cysteine residues which form three intramolecular disulfide bonds (Hancock et al., 1998). Defensins have been shown to form ion channels on artificial membranes (Boman et al., 1991).

β-defensins are highly cationic and contain 38 to 42 amino acid residues. They can be found in most species including humans, birds, rats, mice, Guinea pigs, rabbits, cattle, and sheep (Diamond et al., 2000, Huttner et al., 1998b, Selsted et al., 1996). In mammals β-defensins are one of the two main antimicrobial peptide families (Huttner et al., 1998b). They have been found to be expressed from both epithelial and circulating phagocytic cells (Huttner et al., 1998a). It is believed that β-defensins help to protect the respiratory tract and other mucosal surfaces. The first member of the β-defensin family of mammalian antibacterial peptides discovered was Tracheal Antimicrobial Peptide (TAP) (Diamond et al., 2000). The 38 amino acid residue-long TAP was found to be expressed from the ciliated epithelium of the bovine trachea. Researchers found that it

had a broad spectrum of antibacterial activity. Furthermore, *in vitro* studies showed that cultured tracheal cells exposed to either bacteria or bacterial LPS could greatly increase TAP mRNA synthesis (Diamond et al., 2000). All cattle produce at least 15 different forms of β-defensins (Huttner et al., 1998a). Bactenecins, another antimicrobial peptide, have also been found in bovine neutrophils (Boman et al., 1991).

In addition to β-defensins, the cathelicidins are one of the two principle antimicrobial peptide families in mammals (Huttner et al., 1998b). Cathelicidins have been found in humans, mice, pigs, cattle, and sheep (Hancock et al., 1998, Huttner et al., 1998b). Neutrophil precursor (myeloid) cells of mammals contain the precursor polypeptide (Hancock et al., 1998). The polypeptide is made up of a conserved NH_2-terminal precursor (cathelin) domain of about 100 amino acid residues, immediately followed by an α-helical antimicrobial peptide of about 23 to 38 amino acid residues. The mature peptides are only processed after the neutrophil becomes activated (Storici et al., 1996). The secondary structure of cathelicidin-derived antimicrobial peptides is a cationic, amphipathic, α-helical NH_2-terminus and a straight hydrophobic COOH-terminus (Skerlavaj et al., 1996). Circular dichroism has shown that less than 20% of the structure is in an α-helical conformation, while 30 to 50% of the structure is in an extended (tail) conformation (Storici et al., 1996). Unlike cecropins, cathelicidins contain four cysteine residues which form two disulfide bonds.

More than 20 cathelicidins have been identified (Skerlavaj et al., 1996). Not only have they been shown to have antibacterial properties, but some have been shown to bind bacteria LPS (Hancock et al., 1998). Two bovine cathelicidins were shown to have potent and broad spectrum antibacterial activity *in vitro* (Skerlavaj et al., 1996).

However, both were cytotoxic to human erythrocytes and neutrophils at high concentrations.

Lactoferricins are relatively new antimicrobials which are cleaved from the NH_2-terminus of lactoferrin, an iron-binding protein (Haukland et al., 2001). Lactoferricin B is derived from acid-pepsin hydrolysis of bovine lactoferrin (Haukland et al., 2001, Jones et al., 1994). The primary structure contains 25 amino acid residues, many of them hydrophobic and cationic. Primary sequence analysis suggests that the peptide may interact with biological membranes (Haukland et al., 2001). Lactoferricin B is believed to disrupt the cytoplasmic membrane of bacteria. Researchers have shown that lactoferricin B is rapidly bactericidal against a broad spectrum of bacteria (Jones et al., 1994). However, *Proteus* ssp., *Pseudomonas cepacia,* and *Serratia* ssp. were resistant. Furthermore, variables such as increased ionic strength, increased bacterial inoculum, acid pH, the addition of 5% whole cow's milk, and increasing concentrations of mucin all inhibited or reduced lactoferricin B activity (Jones et al., 1994). Results indicate that this peptide is only transiently active *in vivo*.

Cecropin B as a Novel Antibiotic

As previously mentioned, antibiotic resistant strains of *M. haemolytica* 1:A are becoming more common (Hartman et al., 1993). Increasing patterns of antibiotic resistance, in this and many other pathogens, are fueling a growing public concern for the use of antibiotics in food animals. It may be beneficial to find novel antibacterial agents that are not susceptible to existing mechanisms of resistance. The very potent broad spectrum antibacterial activity of cecropin B makes it a likely candidate to prevent and/or

treat infection. Since it is highly effective against Gram-negative bacteria (Vaara et al., 1994), it may be useful for eradication of pathogens from mucosal surfaces.

One study showed that cecropins in general are 10 to 30 times (molar terms) more potent against *E. coli* and *Pseudomonas aerugenosa* than defensins and magainins (Vaara et al., 1994). Septic shock is primarily induced by endotoxin (LPS) secreted by Gram-negative bacteria. In an effort to determine the efficacy of antimicrobial agents at reducing variables associated with septic shock, researchers found cecropin B to be very effective (Giacometti et al., 2001). Researchers used mono-dose treatments of cecropins A, B and P_1 in rats infected with $2x10^{10}$ colony forming units (CFU) of *E. coli*. Cecropin B was found to be the most effective at preventing bacterial growth, endotoxemia and mortality (Giacometti et al., 2001). While some antibiotics can induce endotoxemia, cecropin B possesses important anti-endotoxemia activity by binding LPS. This and other studies have demonstrated the possible use of antibacterial peptides as topical or injectible antibiotics against pathogens otherwise resistant to conventional antibiotics (Hancock et al., 1998). The ability of cecropin B to effectively inhibit bacterial colonization and neutralize endotoxin further exemplifies its usefulness as a novel antibiotic. However, cost of production may limit this peptide's practical uses. The most cost efficient peptide synthesis methods employed at the present time are still unreasonably expensive (Hancock et al., 1998).

One possible cost-effective approach would be to transfer the cecropin B gene into the species of interest and let the recipient's biosynthesis mechanisms synthesize the peptide. Most transgenic studies involving the cecropin B gene or genes of cecropin B homologues and analogues have been performed in plants such as tobacco, potatoes, rice

and tomatoes. These studies have met with varying success, but suggest that cecropin B can be expressed at the RNA and protein level, while transgenic plants display some resistance to bacterial infection (Hancock et al., 1998). Florack et al. used several different cecropin B gene constructs made for either expression in the cytosol or for secretion (Florack et al., 1995). Plants transfected with a gene construct containing insect signal peptide expressed higher levels of cecropin B mRNA than gene constructs lacking a NH_2-terminal signal peptide. The highest cecropin B mRNA expression levels were seen in plants transfected with a gene construct containing a plant-gene-derived signal peptide (Florack et al., 1995). Even though cecropin B mRNA could be detected, the plants were still not resistant to bacterial wilt and bacterial wildfire. Furthermore, researchers could not detect the cecropin B peptide. Further analysis showed that the peptide was most likely degraded by endogenous proteases (Florack et al., 1995). Cecropins can effectively lyse most Gram-positive and Gram-negative bacteria at very low concentrations of 0.1 to 5 μM, but degradation due to host peptidases can be a very limiting factor (Sharma et al., 2000). To address this problem, researchers fused the coding sequences of mature cecropin B to the signal peptide sequence of the rice chitinase gene which is known to direct the secretion of chitinase gene product into the intercellular spaces of rice (Sharma et al., 2000). After transfecting rice plants with this gene construct they were able to detect expressed cecropin B mRNA, high levels of intercellular cecropin B peptide, and enhanced resistance to bacterial leaf blight. In yet another study, researchers used a synthetic NH_2-terminus-modified cecropin-mellitin peptide chimera to transfect potato plants (Osusky et al., 2000). Using polymerase chain reaction (PCR) and DNA sequencing they were able to confirm stable incorporation of

the gene construct. Cecropin-mellitin chimera mRNA expression was confirmed by reverse transcriptase-PCR (RT-PCR), and they were able to recover active peptide (Osusky et al., 2000). Following highly stringent challenges these transgenic potato plants exhibited potent bacterial resistance. It seems that researchers are well on the path of developing bacterial resistant transgenic plants.

Very few studies are available describing transgenic mammals expressing cecropin B or cecropin B-like peptides. In a preliminary report researchers using a cecropin B analog to transfect mice found the mice to be resistant to *Brucella abortus*, a bovine pathogen (Hagius et al., 1996). There are many indications that the transgenic approach may be useful in enhancing bacterial resistance in food animals, thereby reducing the use of conventional antibiotics.

CHAPTER 2

IN VITRO SUSCEPTIBILITY OF A VIRULENT STRAIN OF *MANNHEIMIA HAEMOLYTICA* 1:A TO CECROPIN B

Introduction

Mannheimia haemolytica 1:A is a gram-negative bacterium generally regarded as the cause of bovine pneumonic pasteurellosis. While *M. haemolytica* 1:A can be a normal resident of the bovine nasal passage, under times of stress, the bacterium proliferates in the upper respiratory tract (URT). The use of antibiotics to treat pneumonic pasteurellosis has been generally successful (Frank, 1986). However, *M. haemolytica* 1:A is developing an increasing pattern of resistance to drugs commonly used to treat the disease (Hartman and Geryl, 1993). Due to an increasing pattern of antibiotic resistance among many species of bacteria, public concern for the use of antibiotics in food animals is once again on the rise (Ferber, 2003). With the occurrence of antibiotic resistant strains of bacteria, the appropriate use of antibiotics should include exposing *M. haemolytica* 1:A to sufficient concentrations of the antimicrobial agents for an appropriate duration as to minimize the development of resistant strains (Watts et al., 1994).

Cecropins are small, strongly basic proteins, which have a broad spectrum of bactericidal activity (Steiner et al., 1988) but are unable to disrupt eukaryotic cells (Boman et al., 1991). This clearly implicates their possible usefulness as novel antibiotics. Cecropins have been shown to have direct action on bacterial membranes (Steiner et al., 1988), although the exact mechanisms are not yet fully understood. Primary membrane attack is believed to be the cause of both Gram-positive and Gram-negative bacteria lysis (Steiner et al., 1988). Furthermore, binding sufficient amounts of cecropin to form a monolayer is necessary to modify the bacterial membranes. Although cecropin B has a broad spectrum of activity against both

Gram-positive and Gram-negative bacteria, the latter are generally more susceptible (Moore et al., 1996). Cecropin B initially interacts with the highly anionic outer membrane glycolipid, LPS, of Gram-negative organisms (Hancock et al., 1998). The peptide then accesses the cytoplasmic membrane by local disruption of the outer membrane.

Cecropin B was chosen for its availability and efficacy. Studies have demonstrated the possible use of cecropin B as topical or injectable antibiotics against pathogens otherwise resistant to conventional antibiotics (Hancock et al., 1998). It has been shown to possess the broadest bactericidal activity of the cecropin family (Chen et al., 1997). The purpose of this study was to determine, in vitro, the antibacterial efficacy of cecropin B for a virulent strain of *M. haemolytica* 1:A. Also, an attempt was made to determine the stability of cecropin B activity in the bovine nasal mucosa.

Materials and Methods

Preparation of Virulent *M. haemolytica* 1:A Inoculum

A preparation of a virulent *M. haemolytica* 1:A strain, lyophilized in skim milk, was reconstituted with 1 ml of sterile distilled H_2O. The suspension was streaked for isolation on yeast blood agar plates with 10% horse serum (10% YBP) and allowed to incubate for 18-24 hours at 37^{o}C in a candle jar (5% CO_2 atmosphere). Bacterial growth was visually identified as *M. haemolytica* 1:A. Isolated colonies were added to 10 ml of sterile Brain Heart Infusion (BHI) broth (Becton Dickinson and Co., BD Microbiology Systems, Sparks, MD) and incubated for an additional 18-24 hours at 37^{o}C. A 100 µl aliquot of this culture was added to 10 ml of sterile BHI broth and incubated for another 8 hours at 37^{o}C. The concentration was adjusted to approximately 8 x 10^6 colony-forming units (CFU) with sterile BHI broth.

Minimum Inhibitory Concentration (MIC)

MICs were determined according to the procedures outlined by the National Committee for Clinical Laboratory Standards (NCCLS). The broth microdilution method was performed in 96-well plates. BHI broth was used instead of the recommended Mueller-Hinton broth (NCCLS). Although Mueller-Hinton broth is acceptable for the growth of *M. haemolytica* 1:A, BHI broth was chosen to meet specific growth requirements and provide consistent growth. Test wells containing two-fold dilutions of cecropin B were incubated in triplicate. Each test well was inoculated with a final concentration of 5×10^4 CFU of *M. haemolytica* 1:A per well. Each plate included positive control (no cecropin B, in triplicate) and negative control (no *M. haemolytica* 1:A) wells. Due to the expense of Cecropin B (Sigma, Sigma-Aldrich Corporation, St. Louis, MO), tetracycline was used first, to standardize the assay. Results were read both visually and with a Dynatech plate reader (Dynatech Laboratories Inc., Chantily, VA) at 16, 20 and 24 hours of incubation. The MIC was determined as the lowest concentration of the antimicrobial agent, which completely inhibited visible growth.

Statistical Analysis

Data were analyzed by use of two-factor ANOVA.

The Stability of Cecropin B in the Bovine Nasal Mucosa

To determine the stability of cecropin B in the bovine nasal mucosa, nasal secretions were collected using a modified 1 ml pipette bent at a 45^o angle approximately 4 to 5 cm from the end and attached to 3 mm diameter tubing that was connected to a 10 ml evacuated tube by a 16 gauge needle. A vacuum was created in the tube using an electric vacuum pump (General Electric Model 5KH35GN106CX, Benton Harbor, MI). The pipette was inserted into each nostril to aspirate nasal secretions. Five milliliters of sterile Dulbecco's phosphate buffered

saline (duPBS) (pH7.2) was then aspirated through the pipette and collected along with mucus in the tube. Nasal secretions were pooled from three calves.

For the assay, 200 µl of *M. haemolytica* 1:A culture (mid-log-phase, final concentration of 10^5 CFU/ml) were mixed with 200 µl of cecropin B diluted in sterile PBS (pH 7.2) (8 µg/ml), as well as 200 µl of cecropin B diluted in pooled bovine nasal secretions (8 µg/ml). The final concentration of cecropin B in each culture was 4 µg/ml. The two assay cultures were allowed to incubate at 37°C. At time intervals 0, 20, and 60 minutes, the assay cultures were diluted 10^{-1} to 10^{-6}, and plated on 10% YBP. The plates were allowed to incubate at 37°C and colonies were counted at 24 hours. The time intervals of 0, 20, and 60 minutes were arbitrarily chosen.

Results

When visually observed, the MIC of tetracycline was 32 µg/ml as seen in Table 2.1. At 16 hours *M. haemolytica* 1:A growth, as determined by optical density at 630 nm, in the positive control wells was significantly higher ($P<0.05$) than growth in all wells containing tetracycline (Figure 2.1). At 20 and 24 hours *M. haemolytica* 1:A growth in the positive control wells was significantly higher ($P<0.05$) than growth at all concentrations of tetracycline except the lowest concentration of 2 µg/ml. Growth measured in these wells was equal to the growth measured in the positive control wells. Growth measured for inhibiting concentrations 128, 64, and 32 µg/ml was not significantly different between 16, 20, or 24 hours of incubation. Wells containing these inhibiting concentrations did not show a significant increase or decrease from 16 to 24 hours. At 16 hours of incubation in the wells where growth was not inhibited, concentrations 16, 8, 4, and 2 µg/ml, growth significantly increased ($P<0.05$) as the concentration of tetracycline decreased. At 20 and 24 hours growth slightly decreased as the concentration of tetracycline decreased from

16 to 4 µg/ml and then increased significantly (P<0.05) as the concentration of tetracycline decreased from 4 to 2 µg/ml.

Table 2.1. Tetracycline susceptibility as determined by visual growth of *M. haemolytica* 1:A.

Tetracycline Concentration (µg/ml)	Visual Growth		
	16hr	20 hr	24hr
128	-	-	-
64	-	-	-
32	-	-	-
16	+	+	+
8	+	+	+
4	+	+	+
2	+	+	+
(+) control	+	+	+

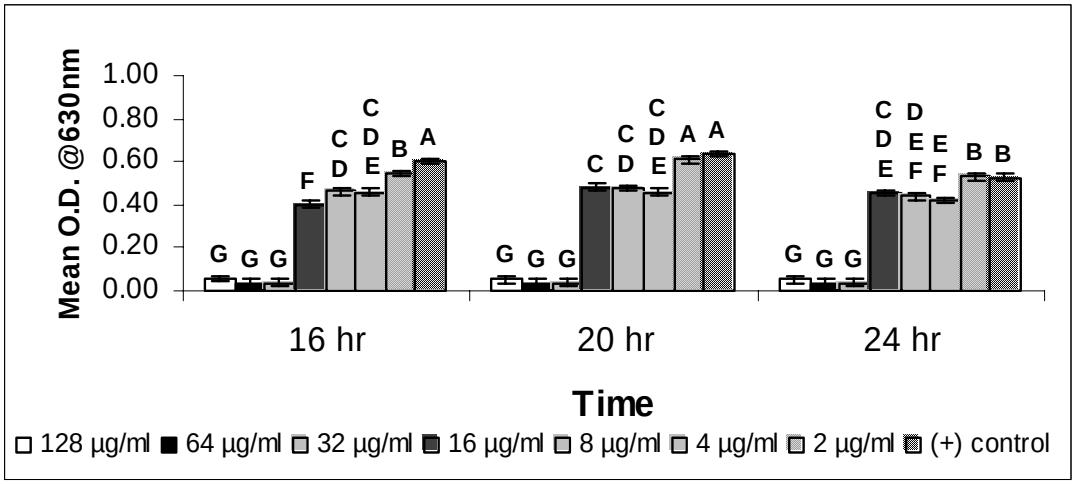


Figure 2.1. Tetracycline susceptibility of *M. haemolytica* 1:A as determined by optical density measured at 630 nm. Optical densities sharing the same letter are not statistically different (P>0.05).

When visually observed, the MIC of cecropin B was 2 µg/ml as seen in Table 2.2. At 16, 20, and 24 hours, *M. haemolytica* 1:A growth, as determined by optical density at 630 nm, in the positive control wells and wells containing 1 µg/ml were significantly higher (P<0.05) than growth in the wells containing 128, 64, 32, 16, 8, 4, and 2 µg/ml of cecropin B (Figure 2.2).

Table 2.2. Cecropin B susceptibility as determined by visual growth of *M. haemolytica* 1:A.

Cecropin B Concentration (µg/ml)	Visual Growth		
	16hr	20 hr	24hr
128	-	-	-
64	-	-	-
32	-	-	-
16	-	-	-
8	-	-	-
4	-	-	-
2	-	-	-
1	+	+	+
(+) control	+	+	+

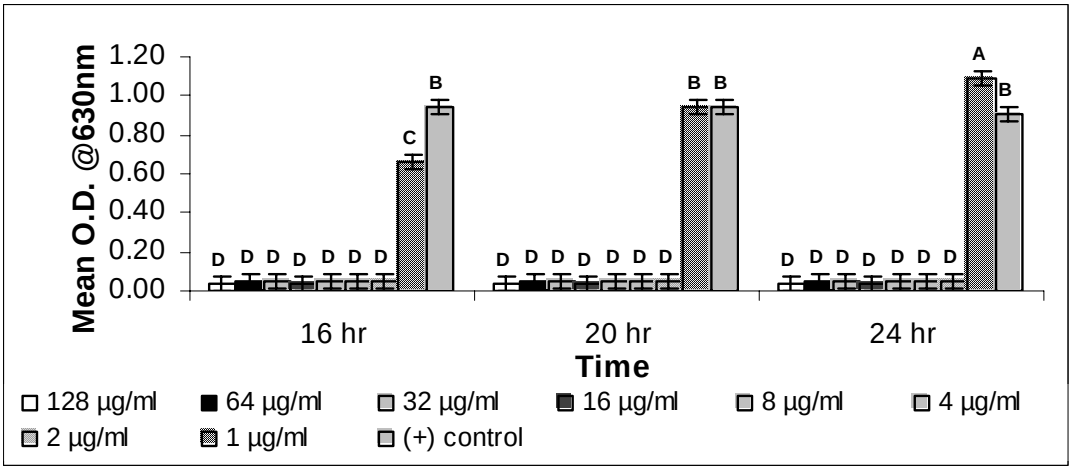


Figure 2.2. Cecropin B susceptibility of *M. haemolytica* 1:A as determined by optical density measured at 630 nm. Optical densities sharing the same letter are not statistically different ($P>0.05$).

Growth measured for concentrations, 128, 64, 32, 16, 8, 4, and 2 µg/ml, was not significantly different from 16 to 24 hours. Wells containing these inhibiting concentrations of cecropin B did not show a significant increase or decrease from 16 to 24 hours. Growth in wells containing 1 µg/ml significantly increased between the 16 and 20 hour readings and again between the 20 and 24 hour readings. At 16 hours of incubation, growth measured in wells containing 1 µg/ml was significantly lower ($P<0.05$) than growth measured in the positive control wells. By 20 hours of

incubation there was no significant difference ($P<0.05$) between these wells. At 24 hours of incubation growth in the wells containing 1 µg/ml of cecropin B was significantly higher than that of the positive control wells. Growth measured in the positive control wells did not increase or decrease from 16 to 24 hours of incubation.

When diluted in PBS (pH 7.2), a 4 µg concentration of cecropin B completely inhibited approximately 10^5 CFU of *M. haemolytica* 1:A within 20 minutes (Figure 2.3). No change was observed at 60 minutes of incubation. When diluted in pooled bovine nasal secretions, a 4 µg concentration of cecropin B again completely inhibited approximately 10^5 CFU of *M. haemolytica* 1:A within 20 minutes (Figure 2.4). Again no change was observed at 60 minutes of incubation.

Discussion

To provide protection against pneumonic pasteurellosis, *M. haemolytica* 1:A should not be allowed to colonize the upper respiratory tract. The use of an effective antibiotic to inhibit the rapid colonization of *M. haemolytica* 1:A in the nasopharynx should reduce the occurrence of pneumonic pasteurellosis in cattle arriving at the feedlots. There have been very few studies aimed at the use of antibiotics to eliminate colonization of the nasal passage. However, in one such study the use of oxytetracycline or feeding chlortetracycline at the feedlots did not reduce or eliminate the colonization of *M. haemolytica* 1:A in the nasopharynx (Frank et al., 2000b). In calves experimentally infected with *M. haemolytica* 1:A, tilmicosin was effective in alleviating clinical signs and pathology findings of pneumonic pasteurellosis as well as the presence of viable organisms in the lung (Morck et al., 1997). Furthermore, the lung could be protected for up to 72 hours with concentrations well above the MIC for *M. haemolytica* 1:A (Morck et al., 1997). Researchers have also shown that a single dose of tilmicosin was more effective

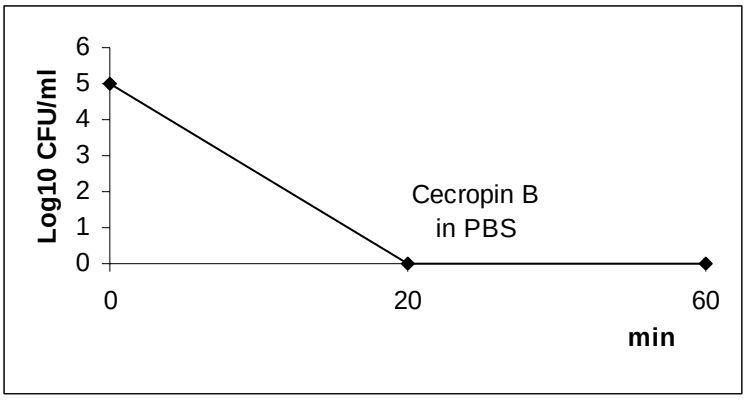


Figure 2.3. Antibacterial activity of cecropin B against *M. haemolytica* 1:A. Mid-log-phase *M. haemolytica* 1:A cells (10^5 CFU/ml) were incubated with cecropin B (4 µg/ml) for 60 minutes at 37°C.

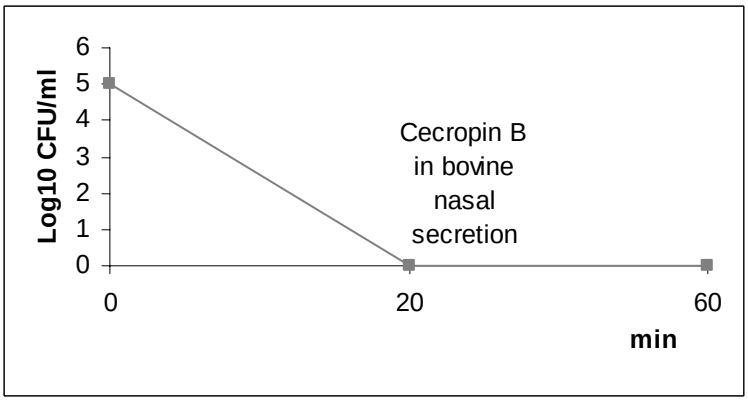


Figure 2.4. Antibacterial activity of cecropin B against *M. haemolytica* 1:A in the presence of pooled bovine nasal secretions. Mid-log-phase *M. haemolytica* 1:A cells (10^5 CFU/ml) were incubated with cecropin B (4 µg/ml) for 60 minutes at 37°C.

at reducing mortality due to pneumonic pasteurellosis than ceftiofur, oxytetracycline, or trimethoprim-sulfadoxine (Shyrock et al., 1996). In other studies focused on decreasing *M. haemolytica* 1:A colonization in the nasopharynx, both tilmicosin and florfenicol were shown to be effective (Frank et al., 2000a, Frank et al., 2002). Both antibiotics inhibited *M. haemolytica* 1:A colonization in the nasal passage for up to several days (Frank et al., 2002).

In this study the MIC of tetracycline was determined as a quality control. The MIC was measured to be 32 µg/ml. This value is consistent with that obtained in a recent study which

showed the MIC of tetracycline for 90% of the *M. haemolytica* 1:A isolates tested to be 32 µg/ml (Watts et al., 1994).

Results of the present study indicate that cecropin B is very effective in inhibiting this virulent strain of *M. haemolytica* 1:A. Growth of this isolate was inhibited at the MIC of 2 µg/ml. However, only one isolate was tested. These results may warrant further testing with larger numbers of isolates to more accurately determine susceptibility breakpoints for *M. haemolytica* 1:A.

The very potent broad spectrum antibacterial activity of cecropin B makes it a likely candidate to prevent and/or treat infection. Since it is highly effective against Gram-negative bacteria (Vaara et al., 1994), it may be useful for eradication of pathogens from mucosal surfaces. One problem to this approach is that the peptide may be degraded by endogenous proteases (Florack et al., 1995, Sharma et al., 2000). While cecropin B can effectively lyse bacteria at very low concentrations, degradation can be a limiting factor. Although proteolytic enzymes in the bovine nasal mucosa may degrade the peptide and inhibit antibacterial activity, no inhibition was observed by incubating cecropin B in bovine nasal secretion. Furthermore, cecropin B was able to completely inhibit *M. haemolytica* 1:A growth within 20 minutes of incubation. The ability of cecropin B to effectively inhibit bacterial colonization as well as neutralize endotoxin further exemplifies its usefulness as a novel antibiotic. However, cost of production may be yet another factor that will limit this peptide's practical uses. The most cost efficient peptide synthesis methods employed at the present time are still unreasonably expensive (Hancock et al., 1998). Therefore, the use of cecropin B as a topical or injectable antibiotic may not be a suitable approach.

CHAPTER 3

TRANSGENIC EXPRESSION OF CECROPIN B IN THE NASAL PASSAGE OF CATTLE CHALLENGED WITH A VIRULENT STRAIN OF *MANNHEIMIA HAEMOLYTICA* 1:A

Introduction

The major etiologic agent of bovine pneumonic pasteurellosis is *Mannheimia haemolytica* 1:A (Dalgleish, 1990, Frank, 1986). Vaccines which provide cattle with effective and consistent immunity to pneumonic pasteurellosis are not yet available. Most veterinarians rely on antimicrobial agents to treat this disease. However, studies have shown *M. haemolytica* 1:A to have an increasing pattern of resistance to many of the commonly used antibiotics (Hartman et al., 1993).

The diapausing pupae of the saturniid *Hyalophora cecropia*, also known as the giant silk moth, respond to bacterial infection with the synthesis of highly effective antibacterial peptides (Boman et al., 1991). Although the moth synthesizes three different classes of antibacterial peptides, the cecropins have the most effective bactericidal activity (Boman et al., 1987, Chen et al., 1997). Cecropin B has a broad spectrum lytic activity, and is very effective against Gram-negative organisms (Vaara et al., 1994).

The very potent antibacterial activity of cecropin B makes it a likely candidate to prevent and/or treat infection. Since it is highly effective against Gram-negative bacteria, it may be useful for eradication of pathogens from mucosal surfaces. Few studies are available describing transfected mammals expressing cecropin B or cecropin B-like peptides; however there are many indications that a transgenic or gene therapeutic approach may be useful in enhancing bacterial resistance in cattle.

Materials and Methods

Transposon-based Plasmid Vector

A transposon-based plasmid vector containing the cecropin B gene was obtained from Dr. Richard Cooper (Cooper, 1998, Cooper and Enright, 1996). This plasmid contains the cecropin B gene linked to an acute phase response promoter (APR) was inserted between 2 transposon insertion sequences. The plasmid also contained the Ptac promoter for the transcription of transposase (Ats). Ats was also encoded on the plasmid. The plasmid was delivered by aerosol while complexed to a transfection reagent (Superfect®, Qiagen, Inc., Valencia, CA). Superfect® is designed to deliver DNA to the nucleus of eukaryotic cells. It is a specifically designed activated dendrimer. The reagent complexes with DNA and arranges it in compact structures to ease entry into the cell. Superfect® complexed with the plasmid possesses a net positive charge, which allows it to bind negatively charged cell surface receptors. The reagent also stabilizes plasmid DNA inside the cell by pH inhibition of lysosomal nucleases. The plasmid was complexed with Superfect® at a 1:3 ratio.

The aerosol was administered using methods derived from a previous study (Brennan et al., 1998). The delivery system for the aerosol was a modified siphon-feed gun (Sears Craftsman Model 491.167060, Hoffman Estates, IL.) with a 7 cm piece of tubing attached to the nozzle. The gun was attached to a nitrogen tank and 20 kg of pressure was used to intranasally aerosolize the suspension. All personnel involved in the DNA delivery were wearing disposable coveralls, disposable latex gloves and HEPA filtered respirators to prevent accidental exposure. The disposable coveralls and latex gloves were properly disposed as biohazardous waste.

Experiment I

Seven (n=7) 300-500 lbs. crossbred beef calves were brought to the Louisiana State University Veterinary Science Farm in Baton Rouge, LA and kept on pasture. Calves were randomly assigned to one of 2 groups. One group was designated the treatment group (n=4), and the other was designated the control group (n=3). The two groups were not isolated during the experiment. Nasal swabs were collected from all calves prior to transfection. Each of the 4 treated calves were given 50 μg of DNA complexed with a transfection reagent (Superfect®, Qiagen, Inc., Valencia, CA) suspended in 3 ml of sterile phosphate buffered saline (PBS) (pH 7.2) per nostril. Control calves were given only the transfection reagent suspended in 3 ml of sterile PBS (pH 7.2) per nostril. Control calves were aerosolized first then allowed to return to the pasture. Next the treated calves were aerosolized and allowed to return to pasture. Nasal swabs were collected on 0, 3, 7, 14, and 21 days post transfection and submitted for DNA polymerase chain reaction (PCR).

Experiment II

Eight (n=8) 300-500 lbs. crossbred beef calves were brought to the Louisiana State University Veterinary Science Farm in Baton Rouge, LA and kept on pasture. Calves were randomly assigned to one of 2 groups. One group was designated the treatment group (n=4), and the other was designated the control group (n=4). The two groups were isolated throughout the experiment. Nasal swabs, nasal aspirates and blood were collected from all calves prior to transfection. Each of the 4 treated calves were given 25 μg of DNA complexed with a transfection reagent (Superfect®, Qiagen, Inc., Valencia, CA) suspended in 3 ml of sterile PBS (pH 7.2) per nostril. Control calves were

given only the transfection reagent suspended in 3 ml of sterile PBS (pH 7.2) per nostril. Control calves were aerosolized first then allowed to return to the pasture. The treated calves were aerosolized one at a time, with that calf being the only calf allowed in the chute at the time. Control calves were isolated from this event. All 8 (treated and control) calves were then inoculated intranasally with 2 x 10^7 to 2 x 10^8 colony forming units (CFU) of a virulent strain of *M. haemolytica* 1:A suspended in 3 ml of sterile Dulbecco's PBS (duPBS) (pH 7.2) (Sigma Chemical Co., St. Louis, MO) per nostril. Following the inoculation, groups were trucked separately 100 miles for 3 hours to stress them. Serum, nasal aspirates, and nasal swabs were collected on 0, 3, 7, 14, 21, and 28 days post transfection/challenge. Samples were analyzed by bacterial culture, real-time PCR, real-time reverse transcriptase (RT) PCR, and ELISA. All samples on day 0 were collected prior to any calf receiving the DNA aerosol. All calves were examined daily for clinical signs of respiratory disease to eliminate the possibility of an adventitious infection.

Experiment III

Twelve (n=12) 300-500 lbs. crossbred beef calves were brought to the Louisiana State University Veterinary Science Farm in Baton Rouge, LA and kept on pasture. Calves were randomly assigned to one of 3 groups. Two groups were designated the treatment groups (n=4 each), and the other was designated the control group (n=4). The three groups were again isolated throughout the experiment. Nasal swabs, nasal aspirates, and blood were collected from all calves prior to transfection. Each of the 4 treated calves in one group were given 50 µg of DNA complexed with a transfection reagent (Superfect®, Qiagen, Inc., Valencia, CA) suspended in 3 ml of sterile PBS pH 7.2

per nostril, as previously described. Each of the 4 treated calves in the other treatment group were given 100 µg of DNA complexed with a transfection reagent (Superfect®, Qiagen, Inc., Valencia, CA) suspended in 3 ml of sterile PBS (pH 7.2) per nostril. Control calves were given only the transfection reagent suspended in 3 ml of sterile PBS (pH 7.2) per nostril. Control calves were aerosolized first then allowed to return to the pasture. The treated calves were aerosolized one at a time, with that calf being the only calf allowed in the chute at the time. The two treated groups were aerosolized on different days. Control calves were isolated from this event. All 12 (treated and control) calves were inoculated intranasally with 2 x 10^7 to 2 x 10^8 CFU of a virulent strain of *M. haemolytica* 1:A suspended in 3 ml of sterile duPBS (pH 7.2) (Sigma Chemical Co., St. Louis, MO) per nostril. Following the inoculation, groups were trucked separately 100 miles for 3 hours to stress them. Serum, nasal aspirates and nasal swabs were collected on 0, 3, 7, 14, 21, and 28 days post transfection/challenge. Samples were analyzed by bacterial culture, real-time PCR, real-time RT-PCR, and ELISA. All samples on day 0 were collected prior to any calf receiving the DNA aerosol. All calves were examined daily for clinical signs of respiratory disease to eliminate the possibility of an adventitious infection.

Preparation of a Virulent *M. haemolytica* 1:A Strain

A preparation of a virulent *M. haemolytica* 1:A strain, lyophilized in skim milk, was reconstituted with 1 ml of sterile distilled H_2O. The suspension was streaked for isolation on yeast blood agar plates with 10% horse serum (10% YBP) and allowed to incubate for 18-24 hours at 37°C in a candle jar (5% CO_2 atmosphere). Bacterial growth was visually identified as *M. haemolytica* 1:A. Isolated colonies were picked and added

to 1 ml of sterile duPBS (pH 7.2) until the solution became turbid. The solution was then used to streak sixteen 10% YBP confluently which were incubated for an additional 18-24 hours at 37°C. Bacterial growth was harvested using a sterile cotton swab and suspended in 20 ml of sterile duPBS (pH 7.2). The concentration was adjusted to approximately 2 x 10^7 to 2 x 10^8 CFU with sterile duPBS (pH 7.2).

Nasal Aspirate Collection

Nasal aspirates were collected using a modified 1 ml pipette bent at a 45° angle approximately 4 to 5 cm from the end and attached to 3 mm diameter tubing that, in turn, was connected to a 10 ml evacuated tube by a 16 gauge needle. A vacuum was created in the tube with a vacuum pump (General Electric Model 5KH35GN106CX, Benton Harbor, MI). The pipette was inserted into each nostril to aspirate nasal secretions. Five milliliters of sterile duPBS (pH 7.2) were then aspirated through the pipette and collected along with mucus into the tube.

Serum Sample Collection

Blood samples were collected into plain evacuated tubes, and allowed to clot at room temperature (approx. 20° C) for 2 hours. Then they were centrifuged at 850 x g for 20 minutes (Beckman TJ-6 Centrifuge, Palo Alto, CA).

PCR Amplification

Total DNA was purified from nasal swabs collected on days 0, 3, 7, 14 and 21 of experiment I using Qiagen DNeasy® extraction kits (Qiagen, Inc., Valencia, CA). Forward and reverse primers were designed to target the cecropin B gene. The forward primer Vec1 (5'-CTACGCTGTAACATCCAAACT-3') and reverse primer Vec2 (5'-

CGCTTTGGCTTCGCCTAAAA-3') were selected. These primers amplify a 946-bp region of the cecropin B gene (bases 401 to 1346, GenBank accession no. X07404). The reaction mixture contained a 50 μM concentration of the forward primer and a 50 μM concentration of the reverse primer. PCR's were performed using PTC-100 Programmable Thermal Controller (MJ Research, Inc., Watertown, MA). The thermal cycler program used was 96ºC for 5 minutes, then 35 cycles at 96ºC for 30 seconds, 58ºC for 30 seconds, and 72ºC for 30 seconds, followed by 72ºC for 5 minutes. All PCR products were electrophoresed on a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer, and the DNA was visualized by ethidium bromide staining under UV fluorescence.

Quantitative Real-time PCR and RT-PCR

Forward and reverse primers as well as a fluorescent probe were designed to target the cecropin B gene. The forward primer CB1-5' (5'-TGGGTCGCAACATCAGAAAC-3'), reverse primer CB1-3' (5'-CCTAGCGCTTTGGCTTCG-3'), and fluorescent CB gene probe (5'-FAM-TCGCCGGTCCAGCCTTGACAATA-TAMRA-3') were selected. These primers amplify a 76-bp region inside the transcribed region of the cecropin B gene (bases 1276 to 1334, GenBank accession no. X07404). The reaction mixture contained a 20 μM concentration of the forward primer, a 20 μM concentration of the reverse primer, and a 7 μM concentration of the fluorescent probe. Real-time PCR, RT-PCR, and probe detection were performed using an ABI PRISM 7900 Sequence detection system (PE Applied Biosystems, Perkin-Elmer, Foster City, CA). The standard real-time PCR thermal cycler program used was 50ºC for 2 minutes, 95ºC for 10 minutes, 40 cycles at 95ºC for 15 seconds, and 60ºC for 1 minute. The standard real-time RT-PCR thermal

cycler program used an additional step of 48ºC for 30 minutes to convert RNA into cDNA.

Total DNA was purified from nasal swabs collected on days 0, 3, 7, 14, 21 and 28 of experiments II & III using Qiagen DNeasy® extraction kits (Qiagen Inc., Valencia, CA). The quantitative results were based on a 5-fold dilution of plasmid DNA. Each total genomic DNA extraction was normalized by using an endogenous control (the eukaryotic 18s rRNA gene) as an active reference to adjust for differences in the amount of total DNA added to each reaction mixture. For an individual calf the relative level of cecropin B DNA in each sample (days 3, 7, 14, 21, and 28) was determined as a ratio compared to day 0 (the calibrator). Results were expressed as arbitrary units (AU).

Total RNA was purified from nasal aspirates collected on days 0, 3, 7, 14, 21, and 28 of experiments II & III using Qiagen RNeasy® extraction kits (Qiagen Inc., Valencia, CA). The quantitative results were based on a 5-fold dilution of bovine RNA containing cecropin B mRNA. Each total genomic RNA extraction was normalized by using an endogenous control (the eukaryotic 18s rRNA gene) as an active reference. In order to adjust for differences in the amount of total RNA added to each reaction mixture. For an individual calf the relative level of cecropin B mRNA in each sample (days 3, 7, 14, 21, and 28) was determined as a ratio compared to day 0 (the calibrator). Results were expressed as arbitrary units (AU).

Bacterial Culture

Nasal aspirates collected from 0, 3, 7, 14, 21, and 28 days post transfection/challenge were analyzed by bacterial culture. Aliquots of these samples were diluted in sterile duPBS (pH 7.2) to 1:10, 1:100, and 1:1000 of the original

concentration. Within one to four hours after collection, small amounts (0.1 ml) of each dilution and of the undiluted sample were directly streaked onto separate 1% YBP (Difco, Becton, Dickinson and Co., Sparks, MD.). The plates were allowed to incubate in a 5% CO_2 atmosphere for up to 72 hours at 37^{o}C. The plates were observed for bacterial growth every 24 hours. Isolated colonies of *M. haemolytica* 1:A as well as the calves' bacterial flora were counted, identified, and recorded.

Small amounts (0.1 ml) of the undiluted sample were inoculated into Difco thioglycollate enrichment medium (Becton, Dickinson and Co., Sparks, MD.) plus 0.1% horse serum. The thioglycollate tubes were allowed to incubate for up to 72 hours at 37^{o}C. The tubes were observed for bacterial growth every 24 hours. Observed growth was then recorded and streaked onto 1% YBP, which were incubated in a 5% CO_2 atmosphere for 24 hours at 37^{o}C.

Suspected *M. haemolytica* 1:A colonies were first identified on the basis of colonial morphology. These representative colonies were then streaked onto manitol salt and McConkey agar plates as well as triple sugar iron (TSI), citrate, and urea slants (Difco, Becton, Dickinson and Co., Sparks, MD.). Smears of these representative colonies were also Gram (Difco, Becton, Dickinson and Co., Sparks, MD.) stained. *M. haemolytica* 1:A colonies were identified on the basis of morphology, cultural, and biochemical characteristics of the isolates in pure culture as well as by the presence of hemolysis, agglutination, and oxidase (Difco, Becton, Dickinson and Co., Sparks, MD.) test. All findings were recorded.

Detection of Cecropin B by ELISA

To test for cecropin B in nasal aspirates collected from days 0, 3, 7, 14, 21, and 28 post transfection/challenge of experiment III, flat bottom wells of Immunolon 1 96-well microtitration plates (Dynatech Laboratories, Chantilly, VA) were coated with 100 µl of a 1:2 dilution of each nasal aspirate in 0.05 M sodium carbonate buffer (.03 M $NaHCO_3$ and .02 M $NaCO_3$, pH 9.6) and incubated overnight at 4^oC for fixation. Serial dilutions of a positive control nasal aspirate spiked with 2 µg/ml of cecropin B were included on each plate. The positive controls were serially diluted 1:2 to 1:256 (2 µg/ml to 0.015625 µg/ml). Also included on each plate were several negative control nasal aspirates which were diluted 1:2. Plates were washed 3 times with PBS (pH 7.2) containing 0.05% (v/v) Tween 20 (PBST). Rabbit anti-cecropin B serum (obtained from Dr. William Todd, LSU Dept. of Vet. Science, Baton Rouge, LA.) diluted 1:200 in PBST was added to each well and incubated 1.5 hours at room temperature. After washing 3 times with PBST, the plates were incubated 1.5 hours with 100 µl of alkaline phosphate-conjugated anti-rabbit IgG (Kirkegaard and Perry, Gaithersberg, MD) diluted 1:1000 in PBST. The plates were washed 3 times with PBST and color was developed by adding 100 µl of substrate buffer containing 1 mg/ml *p*-nitrophenyl phosphatase, 10% diethanolamine, and 5 mM $MgCl_2$ (*p*NPP) (pH 9.8) (Kirkegaard and Perry, Gaithersberg, MD). The absorbance was read at 405 nm in a microplate reader (Benchmark Plus microplate spectrophotometer, Bio-Rad Life Science, Hercules, CA).

The amounts of cecropin B in nasal aspirates were determined using regression equations. Using the serially diluted positive controls, log O.D. values were plotted against the log dilution, generating a regression equation for each plate. Sample

concentrations were calculated by substituting its log O.D. value into the equation. Subtracting the mean concentration of the negative controls plus 2 times the standard deviation from the sample concentration resulted in a corrected cecropin B concentration. Only corrected cecropin B concentrations were reported.

Detection of Antibodies Against *M. haemolytica* 1:A by ELISA

A whole cell *M. haemolytica* 1:A antigen preparation was obtained using previously described methods (Brennan et al., 1998). The whole cell *M. haemolytica* 1:A antigen was diluted 1:200, in 0.1 M sodium carbonate buffer (.06 M $NaHCO_3$ and .04 M $NaCO_3$). The flat bottom wells of Immunolon 1 96-well microtitration plates were then coated with 100 µl of the antigen dilution. The plates were wrapped in cellophane and incubated overnight at 4°C for fixation. After 24 hours of incubation, the plates were washed three times with dH_2O + NaCl + Tween 20, then blocked with 100 µl NET buffer (0.1M NaCl, 1mM EDTA, and 10mM Tris, pH 8.0) plus 10% heat inactivated goat serum per well for 30 minutes at 37°C. The plates were washed as before and 100 µl of each serum sample, diluted 1:200 in NET + 10% goat serum, was added to the appropriate wells. Serial dilutions of a positive control serum sample pooled from high titer calves were included on each plate. The positive controls were serially diluted 1:200 to 1:25,600. Also included on each plate were several negative control serum samples which were diluted 1:200. Plates were then incubated for 1 hour at 37°C and washed as before. Antigen-antibody complexes were detected by adding 100 µl of horseradish peroxidase conjugated affinity purified goat anti bovine immunoglobulins IgG heavy & light chain, IgG_1 heavy chain, IgG_2 heavy chain, IgA-α chain, or IgM-µ chain (Kirkegaard and Perry, Gaithersberg, MD) diluted 1:750 in NET + 10% goat serum per

well. Plates were again incubated for 1 hour at 37°C and then washed. 100 µl of substrate 2-2'azino-di-3-ethylbenzothiazolin sulfone-6 (ABTS) (Kirkegaard and Perry, Gaithersberg, MD) was added to each well and allowed to react at room temperature for ten minutes. The reaction was stopped by the addition of 10% sodium dodecyl sulfate (SDS) (100 µl) per well. Optical densities were read immediately with an ELISA plate reader at a wavelength of 405 nm.

Antibody responses in nasal aspirates were also measured by ELISA similarly to serum samples with a few exceptions. Microtitration plates were coated with antigen overnight as previously described. Nasal aspirates were diluted 1:2 in NET + 10% goat serum. Positive controls were serially diluted 1:2 to 1:256, and negative controls were diluted 1:2.

Antibody titers for serum and nasal aspirates were determined using regression equations. Using the serially diluted positive controls, log OD values were plotted against the log dilution, generating a regression equation for each plate. Sample titers were calculated by substituting its log OD value into the equation. Subtraction of the mean titers of the negative control plus 2 times the standard deviation from the sample titer resulted in a corrected titer. Only corrected titers were reported.

Statistical Analysis

Data were log transferred (natural log) to stabilize variance terms. The SAS statistical package GLM procedure (SAS Institute Inc.) was used to analyze the data through a repeated measures analysis of variance (ANOVA) analyzed as a split-plot arrangement of treatments with 'group' and 'calves within group' as main plot effects and 'day' and 'group by day' as subplot effects. All comparisons were considered

significant at $P<0.05$. Where overall tests indicated significance, least squares mean pairwise t tests for 'group by day' interactions were conducted.

Results

Experiment I

Nasal swabs collected 2 weeks prior to transfection were pooled, while swabs collected on days 0, 3, 7, 14, and 21 were collected from each nostril (right and left) separately. DNA extracted from each swab was submitted to Dr. Richard Cooper's Laboratory (Louisiana State University, Baton Rouge, LA) for PCR. The cecropin B gene was not detected by PCR in any calf 2 weeks prior to transfection (Table 3.1).

Table 3.1. Experiment I PCR results. Pre samples taken 2 weeks prior to day 0 were a pool from both nostrils. All other samples were taken from each nostril.

Calf #	Treatment	Nostril	Day Post Transfection					
			Pre	0	3	7	14	21
139	E50	R	-	-	+	-	+	+
		L		-	-	-	-	+
145	E50	R	-	-	+	+	-	-
		L		+	+	-	-	-
149	E50	R	-	-	-	-	-	+
		L		-	+	+	-	+
151	E50	R	-	+	+	-	-	-
		L		+	+	-	-	-
146	control	R	-	-	-	+	-	-
		L		-	-	-	-	-
148	control	R	-	-	+	+	-	+
		L		-	+	-	-	-
150	control	R	-	-	+	+	+	-
		L		-	+	+	+	-

E50=calves were given 50 μg of plasmid DNA per nostril

Positive PCR results were obtained from both treated calves given 50 μg of plasmid DNA per nostril and control calves after transfection. DNA sequence analysis of PCR products from both treated and control calves showed these sequences to be identical to the corresponding amplified regions of the cecropin B gene (data not shown). Positive

PCR results were obtained from 2 treated calves at day 0 before these calves received the DNA aerosol. Positive PCR results were obtained from all 4 treated and 2 of 3 control calves at day 3. Cecropin B DNA was detected in all 7 calves at some point during the experiment.

Experiment II

Total DNA purified from nasal swabs collected on 0, 3, 7, 14, 21, and 28 days post transfection/challenge was analyzed by real-time PCR. This real-time PCR assay was designed to amplify a 76-bp region of the cecropin B gene. For an individual calf the relative amount of cecropin B DNA in each sample (days 3, 7, 14, 21, and 28) was determined as a ratio compared to day 0 (the calibrator). The relative amounts of cecropin B DNA detected on days 3, 7, 14, 21, and 28 in control calves ranged from 1 to 34.6 AU, while calves given 25 μg of plasmid DNA per nostril ranged from 1 to 159.8 AU (Tables 3.2 and 3.3). For all 4 control calves the amount of cecropin B DNA did not appear to increase after day 0, with one exception (Table 3.2). The amount of cecropin B DNA detected for calf #158 at day 3 was 34.6 times higher than at day 0. Although relative amounts of cecropin B DNA that were slightly higher than day 0 were obtained from all 4 control calves at some point during the experiment, these levels did not appear to be significant. The amount of cecropin B DNA detected for treated calf #150 at day 3 was 159.8 times higher than at day 0 (Table 3.3). Increased amounts of cecropin B DNA were also detected in this calf at day 7 and 28. Calf #157 which was given 25 μg of plasmid DNA per nostril had an increased amount of cecropin B DNA detected at day 3. However, this level (13.0 AU) is considerably lower than that of treated calf #150 (159.8 AU). Two of the 4 treated calves in this experiment had low levels of cecropin B DNA

Table 3.2. Real-time PCR, RT-PCR and isolation data of experiment II control calves. Real-time PCR and RT-PCR results are expressed as arbitrary units (AU).

Calf #	Sample	Day Post Transfection/Challenge					
		0	**3**	**7**	**14**	**21**	**28**
152							
	cecropin B DNA	1.0	1.0	1.0	2.8	1.0	1.1
	cecropin B mRNA	1.0	1.0	1.0	1.0	1.0	1.0
	M. haemolytica						
	isolation	-	+	-	-	-	-
153							
	cecropin B DNA	1.0	1.1	1.5	1.0	2.0	1.0
	cecropin B mRNA	1.0	1.6	ND	1.0	1.0	1.9
	M. haemolytica						
	isolation	-	-	+	-	-	-
155							
	cecropin B DNA	1.0	6.4	1.0	1.0	1.0	2.3
	cecropin B mRNA	1.0	1.0	1.0	1.0	1.0	1.0
	M. haemolytica						
	isolation	-	+	-	-	-	-
158							
	cecropin B DNA	1.0	34.6	3.8	6.5	2.0	1.1
	cecropin B mRNA	1.0	1.0	1.0	ND	1.0	1.0
	M. haemolytica						
	isolation	-	+	+	+	+	-

ND= No data; No RNA could be purified

Table 3.3. Real-time PCR, RT-PCR and isolation data of experiment II calves which received 25 µg of plasmid DNA per nostril. Real-time PCR and RT-PCR results are expressed as arbitrary units (AU).

Calf #	Sample	Day post transfection/challenge					
		0	**3**	**7**	**14**	**21**	**28**
150							
	cecropin B DNA	1.0	159.8	18.8	6	4	37.8
	cecropin B mRNA	1.0	1.0	1.0	1.0	1.0	1.0
	M. haemolytica						
	isolation	-	+	+	+	-	-
157							
	cecropin B DNA	1.0	13.0	2.3	1.4	2.7	3.4
	cecropin B mRNA	1.0	3.9	772.6	2.1	ND	2.8
	M. haemolytica						
	isolation	-	+	-	+	-	+
160							
	cecropin B DNA	1.0	1.4	1.0	1.0	1.0	2.3
	cecropin B mRNA	1.0	1.0	1.0	1.0	1.0	1.0
	M. haemolytica						
	isolation	-	+	+	+	+	+
161							
	cecropin B DNA	1.0	1.0	1.0	1.0	1.0	1.2
	cecropin B mRNA	1.0	1.0	1.0	1.0	1.0	1.0
	M. haemolytica						
	isolation	-	+	+	+	+	-

ND= No data; No RNA could be purified

comparable with the controls. Comparisons of cecropin B DNA detected in calves within the 2 groups indicated significant ($P<0.05$) variation among calves in each group. Comparisons of cecropin B DNA detected between each group indicated that there was not significant ($P<0.05$) variation between the treated and control groups for the entire duration of the experiment nor between each group at a specific day. Since overall tests did not indicate significant variation between control calves and calves given 25 μg of plasmid DNA per nostril no further analyses were performed.

Total RNA purified from nasal aspirates collected on 0, 3, 7, 14, 21, and 28 days post transfection/challenge was analyzed by real-time RT-PCR. This real-time RT-PCR assay was designed to amplify a 76-bp region inside the transcribed region of the cecropin B gene. For an individual calf the relative amount of cecropin B mRNA expression in each sample (days 3, 7, 14, 21, and 28) was determined as a ratio compared to day 0 (the calibrator). Relative amounts of cecropin B mRNA detected on days 3, 7, 14, 21 and 28 in control calves ranged from 1 to 1.9 AU, while calves given 25 μg of plasmid DNA per nostril ranged from 1 to 772.6 AU (Tables 3.2 and 3.3). For all 4 control calves cecropin B mRNA expression did not appear to increase after day 0. For control calf #158 in which increased levels of cecropin B DNA was detected at day 3, no increase in mRNA expression was detected at any point of the experiment. Also, for treated calf #150 no increase in mRNA expression was observed. Increased cecropin B mRNA expression was only observed in one treated calf. The amount of cecropin B mRNA expression detected for treated calf #157 at day 7 was 772.6 times higher than at day 0. No increase in mRNA expression was detected in any other calf given 25 μg of plasmid DNA at any point of the experiment. Comparisons of cecropin B mRNA

expression in calves within groups indicated variation approaching significance ($P<0.05$) among calves in each group. Comparisons of cecropin B mRNA expression in each group and each group by day indicated that there was no significant ($P<0.05$) variation between groups or between groups by day. Once again, since overall tests did not indicate significant variation between control calves and calves given 25 μg of plasmid DNA per nostril no further analyses were performed.

M. haemolytica 1:A was not isolated from any calf at day 0 (Tables 3.2 and 3.3). However, after challenge, *M. haemolytica* 1:A was isolated from all calves at some point during the experiment. *M. haemolytica* 1:A was isolated from 3 of 4 control calves at day 3, but only 2 of 4 calves from days 7 thru 28. *M. haemolytica* 1:A was isolated from all 4 calves given 25μg of DNA per nostril at day 3. The bacterium was isolated from all 4 of these calves at days 7, 14, 21, or 28.

Serum and nasal aspirate titers for all antibody classes were considerably low (Table 3.4). No differences in serum titers for any class of antibodies were noticed between control calves and calves given 25 μg of plasmid DNA per nostril. Also, no differences in nasal aspirate titers for any class of antibodies were noticed between control calves and calves given 25 μg of plasmid DNA per nostril with one exception. Nasal aspirate IgA titers against whole *M. haemolytica* 1:A were detected for 2 of 4 control calves at days 21 and 28, whereas no nasal aspirate IgA titers against whole *M. haemolytica* 1:A were detected for any calf given 25 μg of plasmid DNA per nostril. Again, overall tests indicated no significant ($P<0.05$) differences between the 2 groups or between the two groups at any given day post transfection/challenge for any class of antibody in either serum or nasal aspirates.

Table 3.4. Mean serum and nasal antibody titers for experiment II calves against whole *M. haemolytica* 1:A antigen.

Sample and antibody class			Day Post Transfection/Challenge					
			0	**3**	**7**	**14**	**21**	**28**
Serum								
	IgG(h &l)							
		control	0	0	0.9	0.5	0.5	0.4
		E25	0	0	1.2	0.8	0.4	0.4
	IgG1							
		control	0	0	0	0.1	0.1	0.1
		E25	0	0	0	0.1	0.1	0.1
	IgG2							
		control	0	0	0	0.1	0.1	0.1
		E25	0	0	0	0	0.1	0.1
	IgM							
		control	0	0	0.2	0.1	0.1	0.1
		E25	0	0	0.2	0.1	0.1	0.1
Nasal aspirate	**IgG(h &l)**							
		control	0	0	0	0	0.29	0.16
		E25	0	0	0	0	0	0
	IgG1							
		control	0	0	0	0	0.01	0
		E25	0	0	0	0	0	0
	IgG2							
		control	0	0	0	0	0	0
		E25	0	0	0	0	0	0
	IgM							
		control	0	0	0	0.01	0.01	0.01
		E25	0	0	0	0.01	0	0
	IgA							
		control	0	0	0	0	2.58	1.27
		E25	0	0	0	0	0	0

E25=calves were given 25 μg of plasmid DNA per nostril

* Overall test indicated significant ($P<0.05$) differences between groups, least square mean pairwise t tests for group by day interactions were performed

† Significantly ($P<0.05$) greater than mean titer for controls on that day

All calves were examined daily for clinical signs of respiratory disease to eliminate the possibility of an adventitious infection. However, no evidence of an adventitious infection was observed.

Experiment III

Total DNA purified from nasal swabs collected on 0, 3, 7, 14, 21, and 28 days post transfection/challenge was analyzed by real-time PCR as in experiment II. The relative cecropin B DNA level of days 3, 7, 14, 21, and 28 as compared with day 0 of control calves ranged from 0 to 4.6 AU, while calves given 50 µg of plasmid DNA per nostril ranged from 0 to 988.0 AU (Tables 3.5 and 3.6). The amount of cecropin B DNA detected in calves given 100 µg of plasmid DNA per nostril ranged from 0 to 9983.1 AU (Table 3.7). For all 4 control calves the amount of cecropin B DNA did not appear to increase after day 0 (Table 3.5). For 3 of the 4 calves given 50 µg of plasmid DNA per nostril increases in cecropin B DNA were detected (Table 3.6). For these calves the relative amounts of cecropin B DNA ranged from 100.9 to 988.0 AU. For calves given

Table 3.5. Real-time PCR, RT-PCR and isolation data of experiment III control calves. Real-time PCR and RT-PCR results are expressed as arbitrary units (AU).

Calf #	Sample	Day Post Transfection/Challenge					
		0	**3**	**7**	**14**	**21**	**28**
2189							
	cecropin B DNA	1.0	1.2	1.6	1.0	1.0	1.0
	cecropin B mRNA	1.0	3.2	1.0	1.0	1.0	1.0
	***M. haemolytica* isolation**	-	-	+	-	-	-
2193							
	cecropin B DNA	1.0	4.6	2.6	4.5	1.0	1.0
	cecropin B mRNA	1.0	1.0	1.0	1.0	ND	1.0
	***M. haemolytica* isolation**	-	+	+	-	-	-
2237							
	cecropin B DNA	1.0	2.3	1.0	1.0	1.0	1.0
	cecropin B mRNA	1.0	2.4	1.0	1.0	1.0	1.0
	***M. haemolytica* isolation**	-	-	+	-	-	-
2243							
	cecropin B DNA	1.0	1.0	1.0	1.0	1.0	1.0
	cecropin B mRNA	1.0	1.0	1.0	1.0	ND	1.0
	***M. haemolytica* isolation**	-	-	-	-	-	-

ND= No data; No RNA could be purified

Table 3.6. Real-time PCR, RT-PCR and isolation data of experiment III calves which received 50 µg of plasmid DNA per nostril. Real-time PCR and RT-PCR results are expressed as arbitrary units (AU).

Calf #	Sample	Day Post Transfection/Challenge					
		0	**3**	**7**	**14**	**21**	**28**
2013							
	cecropin B DNA	1.0	1.0	1.0	1.0	1.0	1.0
	cecropin B mRNA	1.0	12.1	2.0	1.0	1.0	1.0
	***M. haemolytica* isolation**	-	+	-	-	-	-
2069							
	cecropin B DNA	1.0	100.9	252.2	2.3	3.4	3.6
	cecropin B mRNA	1.0	148.8	6.4	1.0	ND	19.6
	***M. haemolytica* isolation**	-	-	-	-	-	-
2091							
	cecropin B DNA	1.0	469.0	5.3	1.7	1.5	1.0
	cecropin B mRNA	1.0	7.9	4.3	1.0	1.0	2.3
	***M. haemolytica* isolation**	-	-	-	+	-	-
2231							
	cecropin B DNA	1.0	988.0	1.0	1.0	1.0	1.0
	cecropin B mRNA	1.0	209.1	7.9	1.0	1.0	1.0
	***M. haemolytica* isolation**	-	-	-	-	-	-

ND= No data; No RNA could be purified

Table 3.7. Real-time PCR, RT-PCR and isolation data of experiment III calves which received 100 µg of plasmid DNA per nostril. Real-time PCR and RT-PCR results are expressed as arbitrary units (AU).

Calf #	Sample	Day Post Transfection/Challenge					
		0	**3**	**7**	**14**	**21**	**28**
2012							
	cecropin B DNA	1.0	2.2	2.1	1.0	1.6	1.0
	cecropin B mRNA	1.0	53.1	78.9	1.0	1.0	1.0
	***M. haemolytica* isolation**	-	-	-	-	-	-
2027							
	cecropin B DNA	1.0	1.0	1.0	1.0	1.0	1.0
	cecropin B mRNA	1.0	11.0	1.4	1.0	1.0	1.0
	***M. haemolytica* isolation**	-	-	-	-	-	-
2077							
	cecropin B DNA	1.0	9983.1	4.8	5.0	14.2	13.0
	cecropin B mRNA	1.0	6.4	5.8	1.0	ND	1.0
	***M. haemolytica* isolation**	-	-	-	-	-	-
2178							
	cecropin B DNA	1.0	1.4	1.1	1.1	1.1	1.0
	cecropin B mRNA	1.0	150.7	1.0	2.3	ND	1.2
	***M. haemolytica* isolation**	-	-	-	-	-	-

ND= No data; No RNA could be purified

100 μg of plasmid DNA per nostril, increases in cecropin B DNA were only detected in 1 calf (Table 3.7). At day 3 the relative amount of cecropin B DNA was 9983.1 times higher than day 0 in calf #2077. Comparisons of cecropin B DNA detected in calves within the 3 groups indicated significant (P<0.05) variation among calves in each group. Comparisons of cecropin B DNA detected between each group indicated that there was not any significant (P<0.05) variation between the treated and control groups for the entire duration of the experiment or between each group on a specific day. No further analyses were performed because overall tests did not indicate significant variation between control calves and the 2 treated groups of calves.

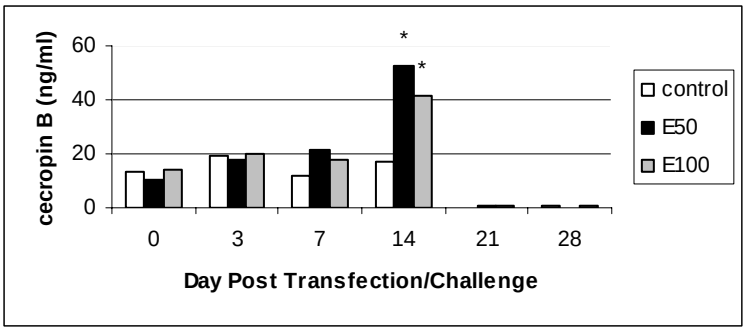
Total RNA purified from nasal aspirates collected on 0, 3, 7, 14, 21, and 28 days post transfection/challenge was analyzed by real-time RT-PCR as in experiment II. For all 4 control calves no increase in cecropin B mRNA expression after day 0 was observed (Table 3.5). Increases in cecropin B mRNA were observed at day 3 in 2 of the 4 calves given 50 μg of plasmid DNA per nostril (Table 3.6). Likewise, increases in cecropin B mRNA were observed at day 3 in 2 of the 4 calves given 100 μg of plasmid DNA per nostril as well (Table 3.7). Comparisons of cecropin B mRNA expression among calves within the three groups indicated no significance (P<0.05) in variation. However, comparisons of mean cecropin B mRNA expression between groups and betweens groups by day indicated that there was significant (P<0.05) variation between the 3 groups. When compared to the control group cecropin B mRNA expression was significantly (P<0.05) increased in both treated groups at 3 and 7 days post transfection/challenge (Table 3.8). Analyses were based on least squares means of the natural log-transformed data, although raw data means were reported.

Table 3.8. Mean cecropin B mRNA (arbitrary units) detected in calves from experiment III. Asterisks indicate significant (P<0.05) differences as determined by least squares means pairwise t test for group by day interactions. Raw means are reported.

Day	Controls	E50	E100
0	1.0	1.0	1.0
3	1.9	94.5*	55.3*
7	1.0	5.2*	21.8*
14	1.0	1.0	1.3
21	1.0	1.0	1.0
28	1.0	6.0	1.1

E50=calves were given 50 μg of plasmid DNA per nostril
E100=calves were given 100 μg of plasmid DNA per nostril

ELISA was used to detect cecropin B in nasal aspirates collected on 0, 3, 7, 14, 21, and 28 days post transfection/challenge. Cecropin B was detected in all calves at day 0 (Figure 3.1). A significant (P<0.05) increase in the mean amount of cecropin B detected in the 2 groups of calves given 50 μg or 100 μg of plasmid DNA per nostril was



E50=calves were given 50 μg of plasmid DNA per nostril
E100=calves were given 100 μg of plasmid DNA per nostril
* Significantly (P <0.05) greater than mean cecropin B detected for controls at that day

Figure 3.1. Mean cecropin B peptide detection by ELISA. Results are expressed in ng/ml amounts.

observed between days 7 and 14. Furthermore, mean cecropin B detected in calves given 50 μg or 100 μg of plasmid DNA per nostril was significantly (P<0.05) greater than mean amounts detected in the control calves at day 14. Mean amounts of peptide detected in these 2 treated groups at day 14 were not significantly different (P<0.05) from

each other, although slightly higher amounts of cecropin B were detected in calves given 50 μg of plasmid DNA per nostril. For all calves cecropin B detection dropped at days 21 and 28. This decrease in peptide was only statistically significant ($P<0.05$) for the 2 treated groups.

M. haemolytica 1:A was not isolated from any calf at day 0 (Tables 3.5, 3.6, and 3.7). However, after challenge *M. haemolytica* 1:A was isolated from 3 of 4 control calves at some point during the experiment (Table 3.5). At day 3 *M. haemolytica* 1:A was isolated from only 1 of 4 (by enrichment media) calves given 50 μg of plasmid DNA per nostril, and from only one other calf in this group on day 14 (Table 3.6). *M. haemolytica* 1:A was not isolated from any of the 4 calves given 100 μg of plasmid DNA per nostril at any point of the experiment (Table 3.7).

Serum and nasal aspirate titers for all antibody classes were considerably higher than those of the experiment II calves (Table 3.9). Serum titers for all classes of antibody and all 3 groups peaked at day 21. Serum antibody titers for all classes against whole *M. haemolytica* 1:A were higher for calves given 50 μg of plasmid DNA per nostril than control calves and calves given 100 μg of plasmid DNA per nostril. No differences in serum titers for any class of antibody were noticed between control calves and calves given 100 μg of plasmid DNA per nostril with one exception. Serum IgG_1 titers against whole *M. haemolytica* 1:A were slightly higher for calves given 100 μg of plasmid DNA per nostril than titers for control calves. The nasal aspirate IgG (h & l), IgG_1, IgG_2, and IgM titers against whole *M. haemolytica* 1:A for the 3 groups were relatively equal. The nasal aspirate IgA titers for control calves appeared to be higher than calves given 50 μg or 100 μg of plasmid DNA per nostril. The nasal aspirate IgA titers against whole

Table 3.9. Mean serum and nasal antibody titers for experiment III calves against whole *M. haemolytica* 1:A antigen.

Sample and antibody class			Day Post Transfection/Challenge					
			0	**3**	**7**	**14**	**21**	**28**
Serum								
	IgG(h &l)*							
		control	0.1	0.1	18.1	41.1	47.4	47.8
		E50	0.1	0.1	84.1†	118.5†	135.1†	122.4†
		E100	0.1	0.1	14.9	49.9	74.2	56.3
	IgG1*							
		control	0.1	0.1	8.8	16.4	25	23.3
		E50	0	0	24.4†	41.8†	63.5†	51.3†
		E100	0	0	8.7	26.1	32.6	25.4
	IgG2							
		control	0	0	1	3.4	9.6	6.2
		E50	0	0	2.8	6.3	14.7	11.6
		E100	0	0	0.1	2.6	9	6.3
	IgM							
		control	0	0	0	0	0	0
		E50	0	0	0	0	0	0
		E100	0	0	0	0	0	0
Nasal aspirate	**IgG(h &l)**							
		control	0.13	0.06	0.03	0.02	0.61	0.36
		E50	0.02	0	0.18	0.04	0.71	0.53
		E100	0.36	0	0.02	0.02	0.38	0.21
	IgG1							
		control	0.01	0.01	0.01	0.01	0.01	0.01
		E50	0	0	0.02	0.01	0.01	0.03
		E100	0	0	0.01	0.01	0.01	0.01
	IgG2							
		control	0.01	0.01	0	0	0.01	0.01
		E50	0	0.01	0	0	0	0.01
		E100	0	0	0	0	0	0
	IgM							
		control	0	0	0.01	0.01	0.01	0.01
		E50	0	0	0.01	0	0.01	0.01
		E100	0	0	0.01	0	0.01	0.01
	IgA							
		control	0.99	1.16	1.21	0.47	5.62	3.15
		E50	0.03	0	0.67	0.46	3.28	0.12
		E100	1.17	0	0	0	0.84	0.36

E50=calves were given 50 μg of plasmid DNA per nostril

E100=calves were given 100 μg of plasmid DNA per nostril

* Overall test indicated significant ($P<0.05$) differences between groups, least square mean pairwise t tests for group by day interactions were performed

† Significantly ($P<0.05$) greater than mean titer for controls on that day

M. haemolytica 1:A for calves given 50 μg of plasmid DNA per nostril were also slightly higher than calves given 100 μg of DNA per nostril.

Overall tests indicated no significant ($P<0.05$) differences between each group or between each group at any given day for any class of antibody in nasal aspirates, but only for IgG_2 and IgM titers in serum. Tests indicated that there was significant ($P<0.05$) variation between groups by day, while variation between groups for the entire experiment were approaching significance. Least squares means pairwise t test for group by day interactions indicated that mean serum IgG (h & l) and IgG_1 titers for calves given 50 μg of plasmid DNA per nostril were significantly ($P<0.05$) greater than mean titers for the control group and the group of calves given 100 μg of plasmid DNA per nostril at 7 thru 28 days post transfection/challenge (Table 3.9). Mean serum IgG (h & l) and IgG_1 titers for calves given 100 μg of plasmid DNA per nostril were not significantly ($P<0.05$) different than mean titers for the control group at any point of the experiment.

All calves were examined daily for clinical signs of respiratory disease to eliminate the possibility of an adventitious infection. Again, no evidence of an adventitious infection was observed.

Discussion

Previous studies have demonstrated the possible use of antibacterial peptides such as cecropin B as topical or injectable antibiotics against pathogens otherwise resistant to conventional antibiotics (Hancock et al., 1998). However, cost of production may limit this peptide's usefulness. The most cost efficient peptide synthesis methods employed at the present time are still unreasonably expensive (Hancock et al., 1998). One possible cost-effective approach would be to transfer the cecropin B gene into the species of

interest, and let the recipient's biosynthesis mechanisms synthesize the peptide. Most gene transfer studies involving the cecropin B gene or genes of cecropin B homologues and analogues have been performed in plants and fish, but also in mice and oysters (Buchanan et al., 2001, Chiou et al., 2002, Dunham et al., 2002, Hagius et al., 1996, Hancock et al., 1998, Sarmasik et al., 2002, Zhang et al., 1998). These studies have met with some success. Most show that cecropin B can be expressed at the RNA and protein level, while displaying some bacterial resistance.

Results of experiment I indicate that the cecropin B gene can be detected in DNA extracted from nasal swabs collected from transfected calves. Furthermore, the cecropin B gene could be detected for at least 3 weeks post transfection. From this data it seems obvious that aerosolization using the previously described methods was successful in depositing the gene into the nasal passage of calves.

Detection of the cecropin B gene at day 0 prior to these calves being transfected and in untreated control calves could implicate the possible presence of a native bovine cecropin or cecropin-like gene. However, transmission of the gene can not be ruled out. Indeed, studies suggest that cecropins may be widespread throughout the animal kingdom (Boman et al., 1991, Hancock et al., 1998, Marshall and Arenas, 2003). A cecropin was found in the upper part of the porcine small intestine (Lee et al., 1989). Cecropins contribute to the animal's innate immunity in every species in which they have been found. As of this study no bovine cecropin has been identified. Many other antimicrobial peptides have been found in cattle including cathelicidins, lactoferricins, and the β-defensin, Tracheal Antimicrobial Peptide (TAP), among others (Diamond et al., 2000, Haukland et al., 2001, Huttner et al., 1998b). Most of these peptides are very

different from cecropins; for example TAP and the cathelicidins contain several cysteine residues which form disulfide bonds. Cecropins are cysteine-free peptides.

It is also possible that the cecropin B gene was transmitted from treated to control calves. No cecropin B DNA was detected in any experiment I calf two weeks prior to transfection. Also, no cecropin B DNA was detected in the 3 control calves on day 0. Swabs were collected from these calves before any aerosolization of plasmid DNA, then the calves were allowed to return to pasture. The 4 treated calves were sampled, then immediately aerosolized with plasmid DNA one at a time with the remaining calves in the chute right behind them. It could be possible that some of the plasmid DNA aerosolized escaped into the air and contaminated subsequent calves. Unfortunately no records were kept as to the order in which calves were sampled and transfected. Following transfection all treated calves were allowed to return to pasture with the controls where transmission could have occurred. To reduce the possibility of transmission, groups in experiments II and III remained isolated from each other. Furthermore, all calves were sampled before aerosolization of plasmid DNA, and treated calves were aerosolized alone in the chute. Researchers wore protective equipment.

The real-time PCR and RT-PCR assays were designed to quantify cecropin B DNA and mRNA detected. The primers and fluorescent dye-labeled probe were designed to amplify a specific 76-bp region of the cecropin B gene. This sequence is inside the transcribed region of the gene. Without a pure control containing a known number of cecropin B gene copies, absolute quantitations could not be performed. Relative quantitations are easy to perform because quantity is expressed relative to some basis sample (i.e. the calibrator). Quantity is determined from the standard curve and

then divided by the quantity of the calibrator. Here, quantitation was based on 5-fold serial dilutions of bovine DNA spiked with the cecropin B gene. For an individual calf the relative quantitation at days 3, 7, 14, 21, and 28 was determined as a ratio compared to day 0. Day 0 results were designated as the calibrator for each calf. Each calf's day 0 quantity was arbitrarily chosen as its calibrator because it would be expected to have the lowest expression level of the target. The day 0 quantities became the 1x sample, therefore arbitrary units (AU) can be read as an *n*-fold increase in either cecropin B DNA or cecropin B mRNA expression. Hence, 3.0 AU of cecropin B mRNA at day 3 would indicate that cecropin B mRNA expression was 3.0 times higher than at day 0. While 300.0 AU of mRNA at day 3 would indicate that cecropin B mRNA expression was 300.0 times higher than at day 0.

The measurement of cecropin B DNA following transfection provided an indication of the longevity of the gene. Following transfection the plasmid was either cleared from the mucosa or delivered to cells where the gene was stably inserted into the genome. In both experiments II and III the cecropin B gene could be detected even at 28 days post transfection. In most cases these DNA levels were comparable with the levels detected in controls. Once again the detection of cecropin B DNA in untreated calves could be due to a native bovine cecropin, therefore it is unclear whether or not levels of cecropin B DNA detected in transfected calves at 4 weeks is a persistence of the transferred gene. Further research may be necessary to determine the longevity of transfection.

In all 3 treated groups of calves there was significant variation in the amounts of cecropin B DNA detected, but no significant differences were observed between treated

groups and their respective control groups. Although increased amounts of cecropin B DNA were detected in some individual calves from each of the 3 treated groups, the data appeared to be statistically insignificant. However, levels of cecropin B DNA detected in those treated calves was noticeably higher than any control calf indicating some difference between control and treated calves. Perhaps using larger numbers of test calves would validate these findings.

Results of experiment II indicate that an aerosol of 25 μg of plasmid DNA per nostril was not enough to consistently transfect calves. An increase in cecropin B mRNA expression was detected in only one calf given 25 μg of plasmid DNA per nostril. Whereas this level of expression was higher than any of the experiment III treated calves, this result appeared to be statistically insignificant. Furthermore, *M. haemolytica* 1:A was isolated more frequently from calves given 25 μg of plasmid DNA per nostril than any other group in experiments II or III. Even if increased mRNA expression went undetected in these calves it did not provide protection against *M. haemolytica* 1:A following challenge.

Results of experiment III mean cecropin B mRNA expression comparisons between the treated and control groups indicated that significant differences existed between the 3 groups. When compared to the control group cecropin B mRNA expression was significantly increased in calves given 50 μg or 100 μg of plasmid DNA per nostril at 3 and 7 days post transfection. The highest levels of cecropin B mRNA were achieved in most calves at day 3, and expression appeared to decline in these calves between days 3 and 7. Analyses were based on least squared means of the natural log-transformed data, although raw data means were reported.

ELISA was then used to detect cecropin B peptide in nasal aspirates collected from experiment III calves. Cecropin B was detected in all calves at day 0. Once again this implies the presence of native bovine cecropin. However, a significant increase in the mean amount of cecropin B detected in the 2 groups of calves given 50 μg or 100 μg of plasmid DNA per nostril was observed at day 14, while no such increase was observed in the control calves. Mean amounts of the peptide detected in these 2 treated groups at day 14 were not significantly different from each other, although slightly higher amounts of cecropin B were detected in calves given 50 μg of plasmid DNA per nostril. For all calves in experiment III, cecropin B peptide detection dropped at days 21 and 28. These suspicious ELISA results were repeated and validated.

Evidence has shown that the cecropin B activity is dependent on the concentration of cecropin (Boman et al., 1991). Binding sufficient amounts of cecropin to form a monolayer is necessary to modify the bacterial membranes (Steiner et al., 1988). Bacteria which are resistant to cecropin still bind large amounts in a non-productive manner, indicating that cecropins act stoichiometrically. The activity of cecropin B is a matter of mass action, and is not catalytic in nature (Steiner et al., 1988). Hence, more peptide is required to kill more bacteria. If mRNA and subsequent peptide production was halted, then a decline in available cecropin B peptide would occur. The drastic decline in cecropin B detection at days 21 and 28 is likely a result of all available peptide being sequestered to kill bacteria, while no more peptide was being produced.

The raw data showed that expression of cecropin B at the mRNA and protein level appeared to be more consistent among calves given 50 μg of plasmid DNA per nostril than among calves given 100 μg of plasmid DNA per nostril. Certainly mean

cecropin B mRNA and peptide expression levels were higher in calves given 50 μg of plasmid DNA per nostril. However these levels were not found to be significantly different from calves given 100 μg of plasmid DNA per nostril. Protection against challenge with a virulent stain of *M. haemolytica* 1:A seemed more apparent in calves given 100 μg of plasmid DNA per nostril where *M. haemolytica* 1:A was not isolated from any calf at any point during the experiment. *M. haemolytica* 1:A was isolated from 2 of the 4 calves given 50 μg of plasmid DNA per nostril. It should be noted that *M. haemolytica* 1:A was not isolated from the 2 calves given 50 μg of plasmid DNA per nostril in which the highest levels of cecropin B mRNA and peptide expression were detected.

Further differences between the 3 groups of experiment III can be seen in serum and nasal aspirate antibody titers against whole *M. haemolytica* 1:A antigen. Serum IgG (h & l), IgG_1, and IgG_2 titers were consistently higher in calves given 50 μg of plasmid DNA per nostril, while these titers detected in control calves and calves given 100 μg of plasmid DNA per nostril did not appear to differ. In contrast higher IgA antibody titers in nasal aspirates were achieved in control calves. This trend was also observed in experiment II where IgA titers were higher in control calves than calves which received 25 μg of plasmid DNA per nostril. In neither experiment were these IgA titers shown to be statistically significant, nevertheless the trend was observed. It would appear that in the absence of peptide, *M. haemolytica* 1:A antigens were more readily available for IgA production. No sound explanation for consistently higher serum IgG (h & l), IgG_1, and IgG_2 antibody titers in calves given 50 μg of plasmid DNA per nostril can be offered. However, mean cecropin B mRNA and peptide expression levels were highest in calves

given 50 µg of plasmid DNA per nostril. Perhaps in these calves inhibition of *M. haemolytica* 1:A eased phagocytosis and enhanced antibody production. However this would not explain why titers detected in control calves and calves given 100 µg of plasmid DNA per nostril did not appear to differ.

Antibody titers against whole *M. haemolytica* 1:A antigen at day 0 (before challenge) were likely the result of prior exposure to *M. haemolytica* 1:A. These findings were consistent with the results of a previous study (Brennan et al., 1998). These titers were not believed to affect the data collected since they were measured to detect differences in antibody production between control and treated groups, not to determine the efficacy of treatments on antibody production.

Very few studies are available describing transfected mammals expressing cecropin B or cecropin B-like peptides. In a preliminary report researchers using a cecropin B analog to transfect mice found the mice to be resistant to *Brucella abortus*, a bovine pathogen (Hagius et al., 1996). There are many indications that a transgenic or gene therapeutic approach may be useful in enhancing bacterial resistance in food animals, thereby reducing the use of conventional antibiotics. Results of this study show that calves transfected with sufficient copies of the cecropin B gene can express cecropin B at the RNA and protein level, while displaying some resistance to *M. haemolytica* 1:A.

CHAPTER 4

CHANGES IN THE NORMAL AND TRANSIENT NASAL FLORA OF CALVES TRANSFECTED WITH A GENE CODING FOR CECROPIN B

Introduction

Although *Pasteurella multocida* and *Haemophilus somnus* have been associated with Bovine Respiratory Disease (BRD), the major etiologic agent of bovine pneumonic pasteurellosis is *Mannheimia haemolytica* 1:A (Dalgleish, 1990, Frank, 1986). *M. haemolytica* serotype 1, biotype A (formally known as *Pasteurella haemolytica* 1:A) is a nonmotile, Gram-negative coccobacilli or rod, and a facultative anaerobe. *M. haemolytica* 1:A is considered to be part of the transient bacterial flora in the upper respiratory tract (URT) of clinically healthy cattle, and can exist in the nasal cavity in very low numbers (Carter et al., 1991, Whiteley et al., 1992).

In addition to *M. haemolytica*, *Pasteurella Multocida*, and *Haemophilus somnus*, the bacteria commonly associated with BRD, the nasal passage of cattle is home to a diverse collection of bacteria that make up the normal and transient flora. Although a complete listing of all the bacteria found in the nasal passage of cattle does not appear to have been reviewed recently, there are some organisms mentioned in the literature. Some prominent bacteria found in the URT include Gram-positive bacteria such as *Micrococcus, Corynebacterium, Staphylococcus, Streptococcus,* and *Bacillus* as well as Gram-negative bacteria such as Pseudomonas, *Actinobacter, Moraxella, Neisseria catarrhalis*, and *Escherichia coli* (Corbeil et al., 1985, Woldehiwet et al., 1990). Other members of the bovine nasal flora may include Gram-positive bacteria such as *Lactobacillus, Streptomyces,* and *Diplococcus pneumoniae* as well as Gram-negative

bacteria such as *Chromobacterium, Achromobacter, Serratia, Flavobacterium, Proteus,* and *Klebsiella* (Magwood et al., 1969). These species of bacteria have been found to start colonizing the URT as early as the calf's first day of life (Woldehiwet et al., 1990). The total bacterial load in the nasal passage increases with age, and is influenced by environmental temperature (Woldehiwet et al., 1990). The purpose of this study was to ascertain whether or not transfecting the URT of cattle with a gene coding for cecropin B would result in a change in the indigenous and transient flora.

Materials and Methods

Nasal aspirates collected from experiment II and III calves on 0, 3, 7, 14, 21, and 28 days post challenge (DPC) were analyzed by bacterial culture. Aliquots of these samples were diluted in sterile duPBS to 1:10, 1:100, and 1:1000 of the original concentration. Within one to four hours after collection, small amounts (0.1 ml) of each dilution and of the undiluted sample were directly plated onto separate 1% yeast blood agar plates (YBP) (Difco, Becton, Dickinson and Co., Sparks, MD.). The plates were allowed to incubate in a 5% CO_2 atmosphere for up to 72 hours at 37^{o}C. The plates were observed for bacterial growth every 24 hours. Isolated colonies of *M. haemolytica* 1:A as well as the calves' bacterial flora were counted, identified, and recorded.

Small amounts (0.1 ml) of the undiluted sample were inoculated into Difco thioglycollate enrichment medium (Becton, Dickinson and Co., Sparks, MD.) plus 0.1% horse serum. The thioglycollate tubes were allowed to incubate for up to 72 hours at 37^{o}C. The tubes were observed for bacterial growth every 24 hours. Observed growth was then recorded and streaked onto 1% YBP, which were incubated in a 5% CO_2

atmosphere for 24 hours at 37^{o}C. Isolated colonies of *M. haemolytica* 1:A as well as the calves' bacterial flora were identified and recorded.

Pure cultures for representative colonies of all macroscopically different types were first identified on the basis of colonial morphology. These representative colonies were then streaked onto manitol salt and McConkey agar plates as well as triple sugar iron (TSI), citrate, and urea slants (Difco, Becton, Dickinson and Co., Sparks, MD.). Smears of these representative colonies were also Gram (Difco, Becton, Dickinson and Co., Sparks, MD.) stained. Bacteria were identified on the basis of morphological, cultural, and biochemical characteristics of the isolates in pure culture. Coccus shaped bacteria were further identified by catalase (H_2O_2) test. Suspected *M. haemolytica* 1:A colonies were further identified by the presence of hemolysis, as well as an agglutination and oxidase (Difco, Becton, Dickinson and Co., Sparks, MD.) test. All findings were recorded.

Results

During the summer months of experiment II, *M. haemolytica* 1:A was not isolated from any calf at day 0 (Tables 4.1 and 4.2). However, after challenge *M. haemolytica* 1:A was isolated from all calves at some point during the experiment. *M. haemolytica* 1:A was isolated from 3 of 4 control calves at day 3, but only 2 of 4 calves from days 7 thru 28. *M. haemolytica* 1:A was isolated from all 4 calves given 25 µg of plasmid DNA per nostril at day 3. *M. haemolytica* 1:A was also isolated from all 4 of these calves at either days 7, 14, 21, or 28.

During the fall months of experiment III, *M. haemolytica* 1:A was not isolated from any calf at day 0 (Tables 4.3-4.5). However, after challenge *M. haemolytica* 1:A

Table 4.1. Summary of bacteria (CFU) isolated on direct media from experiment II control calves.

	CALF #			
DAY	**158**	**153**	**155**	**152**
0	2.5x10^5 *Micrococcus* spp.	7000 *Corynebacterium* spp. 400 *Micrococcus* spp.	6x10^4 *Achromobacter* spp. >1x10^4 *Micrococcus* spp.	6x10^4 *Corynebacterium* spp.
3	**2.5x10^5 *Mannheimia haemolytica****	2750 *Corynebacterium* spp. 100 *Bacillus* spp.	**1.9x10^4 *M. haemolytica*** 300 *Micrococcus* spp.	**7x10^4 *M. haemolytica**** 1.1x10^5 *Micrococcus* spp. 2000 *Streptococcus* spp.
7	3.1x10^4 *Corynebacterium* spp. **2.1x10^5 *M. haemolytica***	1000 Gram (-) coccus** **1250 *M. haemolytica***	1050 *Corynebacterium* spp. 100 *Staphylococcus* spp.	No isolations
14	100 *Bacillus* spp. 100 *Escherichia* spp. **3000 *M. haemolytica**** 100 *Micrococcus* spp.	1000 *Bacillus* spp. 1000 *Escherichia* spp. 1x10^4 *Micrococcus* spp. 4500 *Streptococcus* spp.	No isolations	No isolations
21	300 *Bacillus* spp. **3.5x10^4 *M. haemolytica*** 5500 *Streptococcus* spp.	4000 *Corynebacterium* spp. 3650 *Streptococcus* spp. 5x10^4 *Streptomyces* spp.	1000 *Bacillus* spp. 400 *Corynebacterium* spp. 100 *Escherichia* spp.	600 *Bacillus* spp. 2x10^4 *Escherichia coli* 7133 *Streptococcus* spp.
28	7000 *Achromobacter* spp. 200 *Micrococcus* spp.	1x10^4 *Corynebacterium* spp.	100 *Achromobacter* spp. 300 *Micrococcus* spp.	300 *Bacillus* spp. 800 *Micrococcus* spp.

*Also isolated from enrichment media
**Unable to identify

Table 4.2. Summary of bacteria (CFU) isolated on direct media from experiment II calves that were given 25 µg of plasmid DNA per nostril.

	CALF #			
DAY	**150**	**157**	**160**	**161**
0	4.3x10^5 *Acinetobacter* spp.	2.8x10^6 *Pseudomonas* spp.	1x10^6 *Micrococcus* spp. 1x10^5 *Pseudomonas* spp.	1.2x10^5 *Corynebacterium* spp. >1x10^6 *Pseudomonas* spp.
3	1000 *Corynebacterium* spp. **1x10^5 *Mannheimia haemolytica*** 2000 *Micrococcus* spp.	**5.8x10^4 *M. haemolytica****	**1.6x10^4 *M. haemolytica****	**1.6x10^4 *M. haemolytica*** 3200 *Micrococcus* spp. 100 *Staphylococcus* spp.
7	2.1x10^4 *Corynebacterium* spp. 4x10^5 *Escherichia* spp. **2.8x10^5 *M. haemolytica***	3x10^4 *Achromobacter* spp. 1000 *Corynebacterium* spp. 2.3x10^4 *Lactobacillus* spp. 1.2x10^4 *Staphylococcus* spp.	**4000 *M. haemolytica***	4x10^4 *Escherichia coli* **2.9x10^5 *M. haemolytica***
14	3000 *Achromobacter* spp. 1x10^4 Gram (-) coccus** **400 *M. haemolytica**** 5500 *Streptococcus* spp.	**6x10^4 *M. haemolytica**** 100 *Micrococcus* spp.	200 *Bacillus* spp. **3.4x10^4 *M. haemolytica*** 2x10^4 *Micrococcus* spp. 6x10^4 *Streptococcus* spp.	1.4x10^4 *Corynebacterium* spp. **5x10^4 *M. haemolytica*** 2x10^4 *Micrococcus* spp.
21	4x10^4 *Achromobacter* spp. 1000 *Bacillus* spp. 7500 *Escherichia* spp.	4000 *Alcaligenes* spp. 4750 *Escherichia* spp. 200 *Micrococcus* spp.	1.6x10^4 *Bacillus* spp. 6x10^4 *Corynebacterium* spp. 6000 *E. coli* **1.7x10^4 *M. haemolytica***	550 *Bacillus* Spp. 9.4x10^4 *Corynebacterium* spp. 100 *E. coli* **1x10^4 *M. haemolytica****
28	3.2x10^5 *Achromobacter* spp. 650 *Micrococcus* spp.	**3x10^4 *M. haemolytica***	2x10^4 *Achromobacter* spp. **1200 *M. haemolytica****	2.6x10^5 *Corynebacterium* spp. 1050 *Micrococcus* spp.

*Also isolated from enrichment media
**Unable to identify

Table 4.3. Summary of bacteria (CFU) isolated on direct media from experiment III control calves.

	CALF #			
DAY	**2193**	**2189**	**2237**	**2243**
0	500 *Corynebacterium* spp. 100 *Bacillus* spp. 300 *Micrococcus* spp.	4x10^4 *Corynebacterium* spp. 1750 *Bacillus* spp. 4.3x10^4 *Micrococcus* spp.	750 *Corynebacterium* spp. 400 *Bacillus* spp.	6.8x10^4 *Corynebacterium* spp. 400 *Bacillus* Spp.
3	650 *Corynebacterium* spp. **5000 *Mannheimia haemolytica*** 100 *Streptococcus* spp.	2750 *Achromobacter* spp.	2.4x10^5 *Corynebacterium* spp. 1700 *Bacillus* spp. 2200 *Escherichia* spp.	4.6x10^4 *Corynebacterium* spp. 200 *Streptococcus* spp.
7	650 *Achromobacter* spp. 650 *Corynebacterium* spp. **1000 *M. haemolytica***	200 *Achromobacter* spp. 600 *Corynebacterium* spp. **2850 *M. haemolytica***	3x10^4 *Achromobacter* spp. 1.2x10^5 *Corynebacterium* spp. **6.5x10^4 *M. haemolytica*** 600 *Micrococcus* spp.	5x10^4 *Corynebacterium* spp. 200 *Micrococcus* spp. 300 *Streptococcus* spp.
14	400 *Corynebacterium* spp. 700 *Micrococcus* spp.	1200 *Streptomyces* spp.	1.6x10^4 *Corynebacterium* spp. 2000 *Bacillus* spp. 1000 *Micrococcus* spp.	900 *Streptomyces* spp.
21	5200 *Micrococcus* spp.	1.3x10^5 *Micrococcus* spp.	1.1x10^5 *Corynebacterium* spp. 1900 *Micrococcus* spp.	8000 *Corynebacterium* spp. 5300 *Micrococcus* spp.
28	5.5x10^4 *Corynebacterium* spp. 1100 *Staphylococcus* spp.	600 Coliform spp.* 1.1x10^5 *Corynebacterium* spp. 100 *Bacillus* spp. 3x10^4 *Micrococcus* spp.	1.2x10^5 *Corynebacterium* spp. 3350 *Micrococcus* spp.	7.8x10^4 *Corynebacterium* spp. 1.1x10^5 *Micrococcus* spp.

*Unable to identify

Table 4.4. Summary of bacteria (CFU) isolated on direct media from experiment III calves that were given 50 µg of plasmid DNA per nostril.

	CALF #			
DAY	**2231**	**2013**	**2091**	**2069**
0	$1.7x10^4$ *Corynebacterium* pp. 200 *Bacillus* spp. $1.2x10^4$ *Micrococcus* spp.	$5.5x10^4$ *Corynebacterium* spp. 6500 *Micrococcus* spp.	3100 *Corynebacterium* spp. 300 *Bacillus* spp. 2500 *Micrococcus* spp.	300 *Corynebacterium* spp. 400 *Bacillus* spp. 300 *Micrococcus* spp.
3	500 *Achromobacter* spp. $3.7x10^4$ *Corynebacterium* spp. 100 *Bacillus* spp.	2000 *Achromobacter* spp. 1950 *Corynebacterium* spp. 400 *Streptococcus* spp. ***Mannheimia haemolytica****	100 *Corynebacterium* spp. 5350 *Micrococcus* spp. 100 *Streptococcus* spp.	500 *Achromobacter* spp. 100 *Bacillus* spp. 2000 *Micrococcus* spp. 400 *Streptococcus* spp.
7	$1.7x10^4$ *Achromobacter* spp. 5300 *Corynebacterium* spp. 200 *Streptococcus* spp.	$1.6x10^4$ *Corynebacterium* spp. 100 *Micrococcus* spp.	$5.7x10^4$ *Corynebacterium* spp.	2500 *Achromobacter* spp. 1100 *Corynebacterium* spp.
14	9500 *Corynebacterium* spp. 550 *Micrococcus* spp.	$3.1x10^5$ *Corynebacterium* spp. 300 *Micrococcus* spp.	5366 *Corynebacterium* spp. 4050 *Bacillus* spp. **$4x10^4$ *M. haemolytica***	$2.4x10^4$ *Corynebacterium* spp. 100 *Bacillus* spp.
21	$2.2x10^4$ *Corynebacterium* spp. 100 *Micrococcus* spp.	$7.1x10^4$ *Corynebacterium* spp. 200 *Micrococcus* spp.	5500 *Corynebacterium* spp.	2600 *Corynebacterium* spp. 100 *Bacillus* spp.
28	$4.3x10^4$ *Corynebacterium* spp.	$5.3x10^4$ *Corynebacterium* spp.	2200 *Corynebacterium* spp. $4x10^4$ *Micrococcus* spp.	$5.2x10^4$ *Corynebacterium* spp. 100 *Bacillus* spp. 300 *Micrococcus* spp.

*Isolated from enrichment media, but not direct; no colony count
**Also isolated from enrichment media

Table 4.5. Summary of bacteria (CFU) isolated on direct media from experiment III calves that were given 100 µg of plasmid DNA per nostril.

	CALF #			
DAY	**2027**	**2178**	**2077**	**2012**
0	1200 *Corynebacterium* spp. 200 *Bacillus* spp.	$1.6x10^5$ *Achromobacter* spp. $2x10^4$ *Micrococcus* spp.	$8.5x10^4$ *Corynebacterium* spp. 2200 *Escherichia* spp. $4.5x10^4$ *Micrococcus* spp.	1000 *Corynebacterium* spp. 500 *Micrococcus* spp.
3	600 *Achromobacter* spp. $2x10^4$ *Corynebacterium* spp.	700 *Corynebacterium* spp. 300 *Micrococcus* spp.	400 *Achromobacter* spp. 100 *Corynebacterium* spp. $2x10^4$ *Micrococcus* spp.	2950 *Achromobacter* spp. 1550 *Corynebacterium* spp. 100 *Bacillus* spp.
7	$6.5x10^4$ *Corynebacterium* spp. 300 *Micrococcus* Spp.	8400 *Corynebacterium* spp. 1000 *Micrococcus* spp. 100 *Streptococcus* spp.	4000 *Achromobacter* spp. 6750 *Corynebacterium* spp. 100 *Streptococcus* spp.	$1x10^4$ *Corynebacterium* spp. 100 *Bacillus* spp. 100 *Micrococcus* spp. 100 *Streptococcus* spp.
14	5000 *Corynebacterium* spp. 650 *Micrococcus* spp. $3x10^4$ *Pseudomonas* spp.	600 *Corynebacterium* spp. 100 *Micrococcus* spp.	300 *Micrococcus* spp.	$5x10^5$ *Corynebacterium* spp. $1.4x10^4$ *Micrococcus* spp.
21	$3.8x10^5$ *Corynebacterium* spp. 100 *Bacillus* spp.	1050 *Corynebacterium* spp. 100 *Bacillus* spp. 600 *Micrococcus* spp.	7050 *Corynebacterium* spp. 100 *Bacillus* spp.	$1.7x10^5$ *Corynebacterium* spp. 100 *Bacillus* spp. 2400 *Micrococcus* spp.
28	$2.6x10^5$ *Corynebacterium* spp. $1x10^4$ *Bacillus* spp. 1400 *Micrococcus* spp.	$8x10^5$ *Corynebacterium* spp.	1000 *Corynebacterium* spp. 900 *Bacillus* spp. $4x10^5$ *Micrococcus* spp.	$3x10^5$ *Corynebacterium* spp. 100 *Bacillus* spp. 800 *Micrococcus* spp.

was isolated from 3 of 4 control calves at some point during the experiment. *M. haemolytica* 1:A was isolated from only 1 of 4 (by enrichment media) calves given 50 µg of plasmid DNA per nostril at day 3, and only 1 of 4 calves from days 7 thru 28. *M. haemolytica* 1:A was not isolated from any of the 4 calves given 100 µg of plasmid DNA per nostril at any point in the experiment.

After challenge *M. haemolytica* 1:A was isolated a total of 38 times from all five groups. The organism was isolated 28 times on direct media and 10 times in enrichment media. Many times it was isolated on both direct and enrichment media, only once was it isolated solely in enrichment media. *M. haemolytica* 1:A was isolated with a 29% frequency from experiment II control calves and a 63% frequency from calves given 25 µg of plasmid DNA per nostril (Figure 4.1). *M. haemolytica* 1:A was the most frequently isolated organism from calves given 25 µg of plasmid DNA per nostril. However, *M. haemolytica* 1:A was only isolated with a 17% frequency from experiment III control calves and a 8% frequency from calves given 50 µg of plasmid DNA per nostril. Again *M. haemolytica* 1:A was not isolated from any calf given 100 µg of plasmid DNA per nostril.

Bacteria isolated from both summer and fall experiments on direct and enrichment media include *Achromobacter, Pseudomonas, Micrococcus, Corynebacterium, Bacillus, Streptococcus,* and more (Tables 4.1-4.5, Figure 4.1). Escherichia coli was isolated several times from both groups in experiment II, but was never isolated during experiment III. Other Escherichia species were isolated during both experiments, but were isolated more frequently during experiment II (Figure 4.1). An

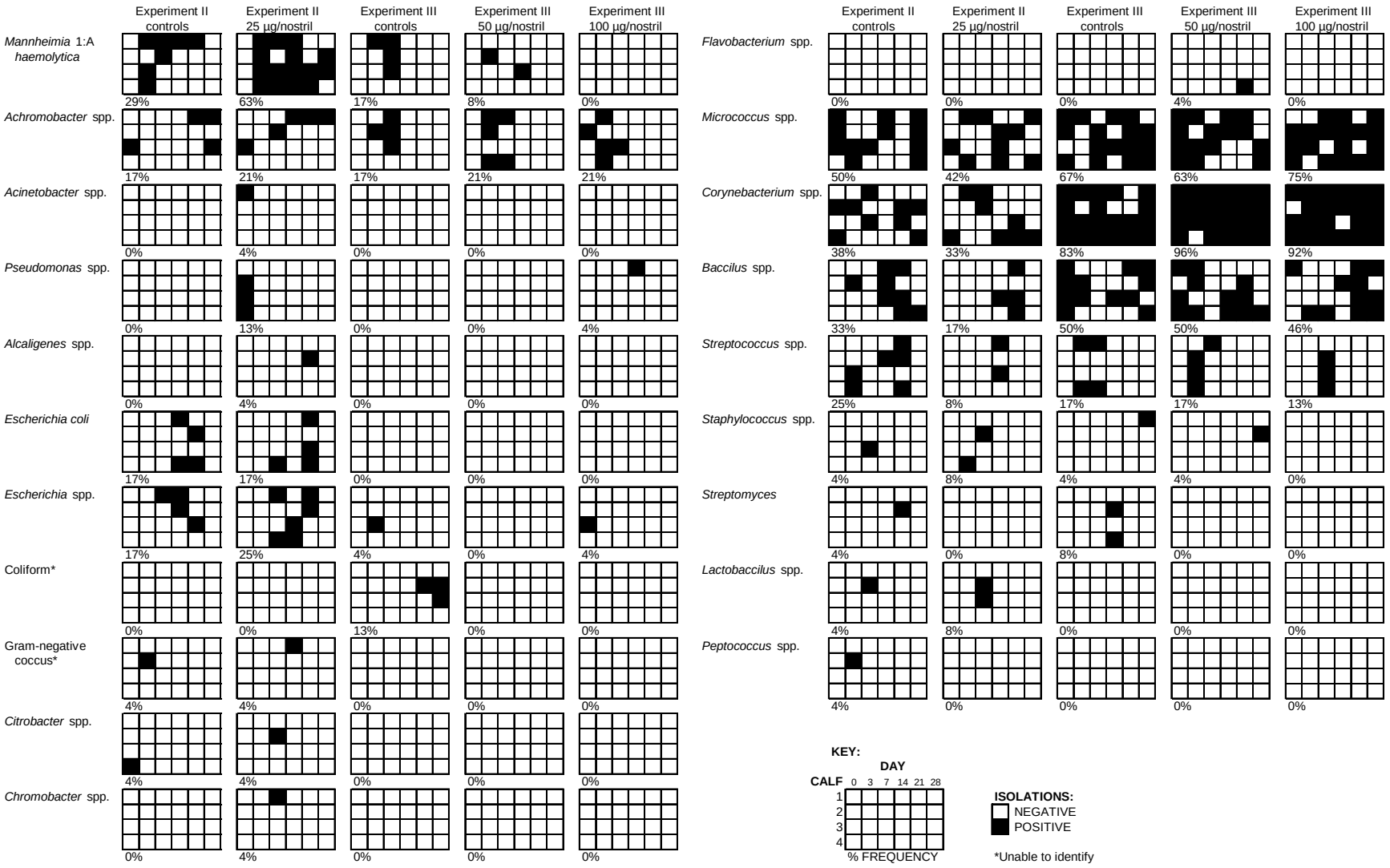


Figure 4.1. Frequency of bacterial isolations from nasal aspirates on direct and enrichment media

unidentifiable Gram-negative coccus was isolated once from an experiment II control calf (Table 4.1) and once from a calf given 25 µg of plasmid DNA per nostril (Table 4.2). An unidentifiable coliform was isolated on direct media once from an experiment III control calf (Table 4.3) and twice on enrichment media from 2 different experiment III control calves.

Percent frequency of the nasal flora isolations were different between experiments II and III, but were consistent among the groups in each experiment (Figure 4.1). *Micrococcus* spp. were the most frequently isolated organisms in experiment II. *Micrococcus* spp. were isolated with a 50% frequency from experiment II control calves and a 42% frequency from calves given 25 µg of plasmid DNA per nostril. In experiment III, *Micrococcus* spp. were isolated with an 67% frequency from experiment III control calves, a 63% frequency from calves given 50 µg of plasmid DNA per nostril and a 75% frequency from calves given 100 µg of plasmid DNA per nostril. *Corynebacterium* spp. were isolated with a 38% frequency from experiment II control calves and a 33% frequency from calves given 25 µg of plasmid DNA per nostril. In contrast, *Corynebacterium* spp. were the most frequently isolated organisms in experiment III. *Corynebacterium* spp. were isolated with an 83% frequency from experiment III control calves, a 96% frequency from calves given 50 µg of plasmid DNA per nostril and a 92% frequency from calves given 100 µg of plasmid DNA per nostril. *Bacillus* spp., *Streptococcus* spp., and *Achromobacter* spp. were also frequently isolated from calves in both experiments.

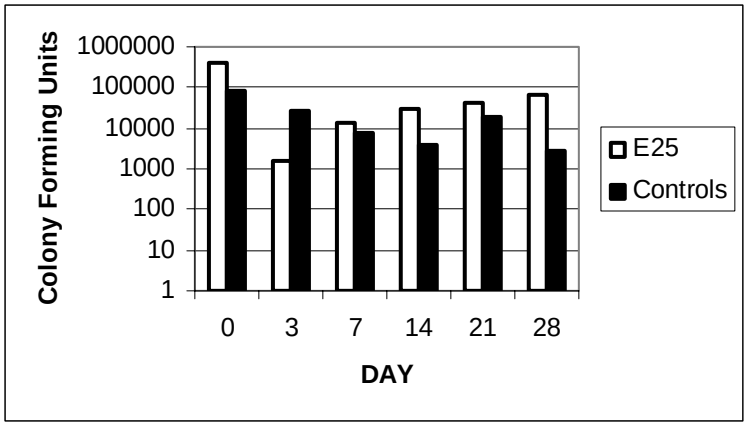
There were no fluctuations in the average amounts of Gram-positive or Gram-negative bacteria (CFU) isolated from either group in experiment II (Table 4.6, Figures

4.2 and 4.3). Also, there were no fluctuations in the average amounts of Gram-positive bacteria (CFU) isolated from any group in experiment III (Table 4.7, Figure 4.4). However, there were drastic fluctuations in the average amounts of Gram-negative bacteria (CFU) isolated from all groups in experiment III (Table 4.7, Figure 4.5).

Table 4.6. Mean Gram-positive bacteria, Gram-negative bacteria and *M. haemolytica* (CFU) per group isolated from experiment II calves on direct media.

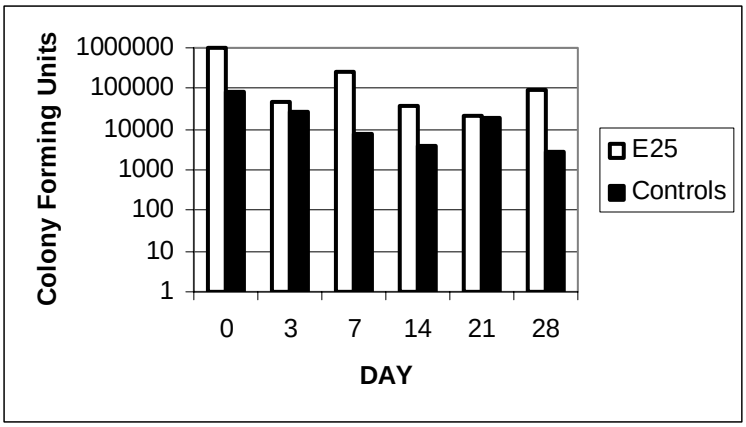
		DAY					
GROUP		0	3	7	14	21	28
E25	Gram (+)	$3.9x10^5$	1575	$1.4x10^4$	$3x10^4$	$4.3x10^4$	$6.5x10^4$
	Gram (-)	$>9.8x10^5$	$4.8x10^4$	$2.6x10^5$	$3.9x10^4$	$2.2x10^4$	$9.3x10^4$
	Mannheimia haemolytica	0	$4.8x10^4$	$1.4x10^5$	$3.6x10^4$	6750	7800
Controls	Gram (+)	$8.2x10^4$	$2.8x10^4$	8038	4050	$1.8x10^4$	2900
	Gram (-)	$1.5x10^4$	$3.1x10^4$	$5.3x10^4$	1025	$1.4x10^4$	1775
	Mannheimia haemolytica	0	$3.1x10^4$	$5.3x10^4$	750	8750	0

E25=calves given 25 µg of DNA per nostril



E25=calves given 25 µg of DNA per nostril

Figure 4.2. Mean Gram-positive bacteria per group isolated from experiment II calves on direct media.



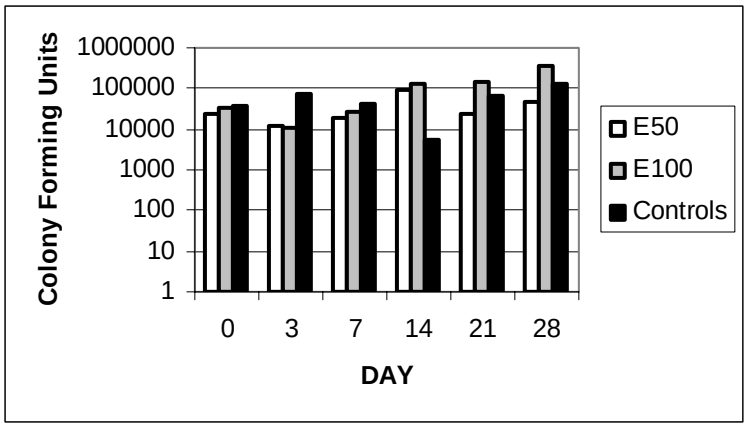
E25=calves given 25 µg of DNA per nostril

Figure 4.3. Mean Gram-negative bacteria per group isolated from experiment II calves on direct media.

Table 4.7. Mean Gram-positive bacteria, Gram-negative bacteria and *M. haemolytica* (CFU) per group isolated from experiment III calves on direct media.

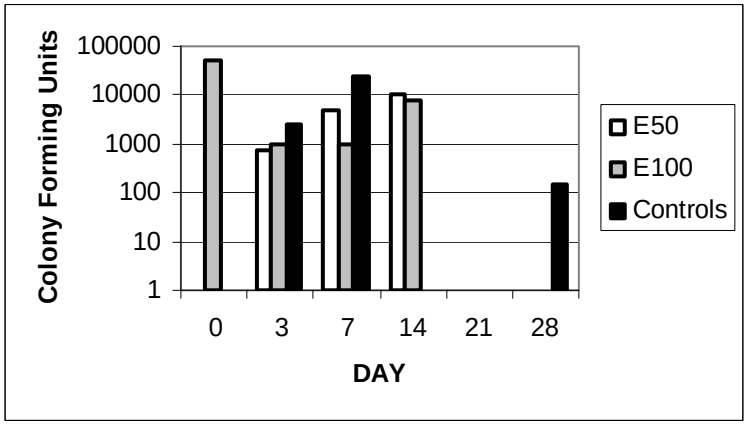
		DAY					
GROUP		0	3	7	14	21	28
E50	Gram (+)	$2.4x10^4$	$1.2x10^4$	$2x10^4$	$8.8x10^4$	$2.5x10^4$	$4.8x10^4$
	Gram (-)	0	750	4875	$1x10^4$	0	0
	Mannheimia haemolytica	0	0	0	$1x10^4$	0	0
E100	Gram (+)	$3.3x10^4$	$1.1x10^4$	$2.8x10^4$	$1.3x10^5$	$1.4x10^5$	$3.8x10^5$
	Gram (-)	$5.3x10^4$	988	1000	7500	0	0
	Mannheimia haemolytica	0	0	0	0	0	0
Controls	Gram (+)	$3.9x10^4$	$7.2x10^4$	$4.3x10^4$	5550	$6.5x10^4$	$1.3x10^5$
	Gram (-)	0	2488	$2.5x10^4$	0	0	150
	Mannheimia haemolytica	0	1250	$1.7x10^4$	0	0	0

E50=calves given 50 µg of DNA per nostril
E100=calves given 100 µg of DNA per nostril



E50=calves given 50 µg of DNA per nostril
E100=calves given 100 µg of DNA per nostril

Figure 4.4. Mean Gram-positive bacteria per group isolated from experiment III calves on direct media.



E50=calves given 50 µg of DNA per nostril
E100=calves given 100 µg of DNA per nostril

Figure 4.5. Mean Gram-negative bacteria per group isolated from experiment III calves on direct media.

On several days during experiment III no Gram-negative bacteria were isolated. Mean Gram-negative bacteria colony counts for control calves dropped to 0 at day 14, but increased slightly by day 28 as seen in figure 4.5. In contrast, mean Gram-negative bacteria colony counts for calves given 50 µg or 100 µg of plasmid DNA per nostril dropped to 0 at day 21, and did not increase by day 28 (Figure 4.5).

In both experiments II and III *M. haemolytica* 1:A was more often isolated with Gram-positive bacteria than with Gram-negative bacteria (Table 4.8). *M. haemolytica* 1:A was isolated with Gram-positive bacteria 84% of the time, while it was isolated with Gram-negative bacteria only 34% of the time. *Corynebacterium* spp. were isolated with *M. haemolytica* 1:A 11 times (29% frequency). Also, *Micrococcus* spp. and *Bacillus* spp. were isolated with *M. haemolytica* 1:A 9 times (24% frequency) and 8 times (21% frequency) respectively.

Table 4.8. Bacteria isolated with *M. haemolytica* 1:A on both direct and enrichment media.

Bacteria	Frequency	Frequency %	Rank
None	5	13%	4
Achromobacter spp.	5	13%	4
Bacillus spp.	8	21%	3
Corynebacterium spp.	11	29%	1
Escherichia spp.	2	5%	6
Escherichia coli	4	11%	5
Gram-negative coccus	2	5%	6
Micrococcus spp.	9	24%	2
Staphylococcus spp.	1	3%	7
Streptococcus spp.	5	13%	4
Gram-positive spp.	32	84%	
Gram-negative spp.	13	34%	

Note: *M. haemolytica* 1:A was isolated 38 times.

Discussion

The major etiologic agent of bovine pneumonic pasteurellosis is *Mannheimia haemolytica* 1:A (Dalgleish, 1990, Frank, 1986), which is believed to be part of the normal bacterial flora in the URT of clinically healthy cattle; and can exist in the nasal cavity in very low numbers (Carter et al., 1991, Whiteley et al., 1992). It is believed that

healthy cattle can carry undetectable levels of *M. haemolytica* 1:A for long periods of time (Frank, 1988). *M. haemolytica* 1:A was not isolated from any calf in either experiment prior to challenge. The organism is likely carried in one or more sites distinct from the nasal cavity, specifically the palatine tonsils (Frank et al., 1992). While remaining in the tonsils, *M. haemolytica* 1:A is available for stress induced colonization of the nasal cavity. In these experiments all calves were stressed, after challenge, by transportation in a crowded trailer, 100 miles for 3 hours. Seven out of the 8 control calves for the 2 experiments yielded detectible levels of *M. haemolytica* 1:A in nasal aspirates throughout the weeks following challenge. Stressed cattle have been shown to shed larger numbers of the bacterium (Frank et al., 1986). Calves aerosolized with 25 µg of plasmid DNA per nostril did not gain any protection against *M. haemolytica* 1:A. All 4 of these calves yielded detectible levels of *M. haemolytica* 1:A in nasal aspirates. Furthermore, *M. haemolytica* 1:A was the most frequently isolated organism from calves given 25 µg of plasmid DNA per nostril. As early as 1969 it was shown that *M. haemolytica* was capable of out numbering the rest of the nasal flora for several days at a time (Magwood et al., 1969). *M. haemolytica* 1:A isolated from control calves and calves given 25 µg of plasmid DNA per nostril also fluctuated in frequency and numbers. Two of the 4 calves given 50 µg of plasmid DNA per nostril yielded detectible levels of *M. haemolytica* 1:A in nasal aspirates following challenge. However, *M. haemolytica* 1:A was not detected in any calf given 100 µg of plasmid DNA per nostril at any point during experiment III. Calves aerosolized with 50 or 100 µg of plasmid DNA per nostril appeared to gain some or total protection to *M. haemolytica* 1:A, respectively.

The nasal passage of cattle is home to a diverse collection of bacteria that make up the normal and transient flora. These species of bacteria have been found to start colonizing the URT as early as the calf's first day of life, and the total bacterial load increases with age, and under the influence of environmental temperature (Woldehiwet et al., 1990). Studies have shown that the population of bacteria in the nasal passage of calves fluctuates heavily in types and numbers (Magwood et al., 1969, Woldehiwet et al., 1990). Sometimes one genus or species is more dominant than others for random periods of time. Here, *M. haemolytica* 1:A was shown to dominate the nasal passages of calves at several collection days during both experiments. Also, fluctuations in the types and numbers of bacteria were observed in individual calves. The percent frequency of nasal flora isolates were different between the summer and fall experiments, but were consistent among the groups in each experiment. These results indicate that these organisms were always present at some level, but may have been increased or reduced due to environmental temperature. *Micrococcus* spp. were the most frequently isolated organisms in experiment II control calves. However, *Micrococcus* spp. were isolated less frequently than *M. haemolytica* 1:A from calves given 25 µg of plasmid DNA per nostril. It is believed that most *Micrococcus* spp. are heavily circulated in the air, and may not be able to colonize the nasal mucosa (Magwood et al., 1969). Since they are regularly isolated from the nasal passage they may still be considered part of the normal flora. More frequent isolation of *Micrococcus* spp. during the summer experiments may have been a result of drier weather. *Corynebacterium* spp. were isolated from all experiment II calves, but not as frequently as *M. haemolytica* 1:A and *Micrococcus* spp. During the fall months of experiment III, *Corynebacterium* spp. were the most frequently isolated

organism from control calves, calves given 50 µg of plasmid DNA per nostril and calves given 100 µg of plasmid DNA per nostril. *M. haemolytica* 1:A and *Micrococcus* spp. were isolated less frequently. *Bacillus* spp. were isolated from calves in both the summer and fall experiments, although more frequently during the fall experiment. In both experiments *Bacillus* spp. were isolated in lower numbers that most other organisms. *Escherichia coli* was isolated several times from both groups in experiment II, but was never isolated during experiment III. Isolation of *Escherichia coli* may actually be the result of fecal material in and around the nostrils.

There appeared to be no significant change in the normal bacterial nasal flora. However, there were drastic fluctuations in the average amounts of Gram-negative bacteria isolated from all groups in experiment III. Mean Gram-negative bacteria colony counts for calves given 50 µg or 100 µg of plasmid DNA per nostril dropped late during the experiment, after which no Gram-negative bacteria were isolated again. Mean Gram-negative bacteria colony counts for control calves also dropped late in the experiment, after which only small amounts of Gram-negative bacteria were detected. It appeared that transfecting the nasal passages of cattle may have affected the Gram-negative nasal flora. However, due to drastic fluctuations in the average amounts of Gram-negative bacteria isolated from all groups in experiment III, including the control calves, transfecting the nasal passages of cattle with a gene coding for cecropin B did not significantly alter the normal indigenous and transient nasal flora.

Bacteria which make up the normal indigenous and transient nasal flora may enhance or inhibit growth of *M. haemolytica*. *Bacillus* isolates were found to inhibit the growth of *M. haemolytica in vitro* (Corbeil et al., 1985). In contrast, several Gram-

positive bacteria such as *Micrococcus*, *Corynebacterium*, and *Staphylococcus* were found to efficiently enhance the growth of *M. haemolytica in vitro* (Corbeil et al., 1985). In this study *M. haemolytica* 1:A was more often isolated with the Gram-positive bacteria, specifically *Corynebacterium* spp. and *Micrococcus* spp., than with Gram-negative bacteria. However, *Bacillus* spp. were isolated with *M. haemolytica* 1:A, although in low numbers. Also, it may be important to note that these three groups of bacteria were the most frequently isolated from all calves in both experiments. Even though studies have shown that the normal and transient flora of the nasal passage can significantly affect the colonization of *M. haemolytica* 1:A (Corbeil et al., 1985), no conclusions could be made from these results.

SUMMARY AND CONCLUSIONS

The nasal passages of calves were aerosolized with different amounts of plasmid DNA containing a gene coding for cecropin B. Results indicate that the cecropin B gene or cecropin B mRNA expression can be detected in DNA or RNA extracted from nasal swabs or nasal aspirates. Furthermore, the cecropin B gene could be detected for at least 4 weeks post transfection. Detection of the cecropin B gene prior to calves being transfected and in untreated control calves could implicate the possible presence of a native bovine cecropin or cecropin-like gene and is a new finding. Since the detection of cecropin B DNA in untreated calves could be due to a native bovine cecropin, it is unclear whether or not levels of cecropin B DNA detected in transfected calves at 4 weeks is a persistence of the transferred gene. Further research may be necessary to determine the longevity of transfection.

Transfecting calves with 50 or 100 µg of plasmid DNA per nostril provided sufficient copies the cecropin B gene to transfect areas of the nasal passage. Transfected areas of the nasal passage expressed cecropin B detectable at the mRNA and peptide level. Results of these experiments indicate that an aerosol of 25 µg of plasmid DNA per nostril was not enough to consistently transfect the nasal passage of calves. However, when compared to the control group cecropin B mRNA expression was significantly increased in calves given 50 µg or 100 µg of plasmid DNA per nostril at 3 and 7 days post transfection. When ELISA was used to detect the peptide in nasal aspirates, a significant increase in mean amounts of cecropin B was observed at day 14 in the two

groups of calves given 50 µg or 100 µg of plasmid DNA per nostril. No such increase was observed in the control calves.

Results of this study also indicate that cecropin B is very effective in inhibiting the virulent challenge strain of *Mannheimia haemolytica* 1:A *in vitro*. Growth of this isolate was inhibited at a concentration of 2 µg/ml. While establishing susceptibility guidelines was not the goal of this study, these results may warrant further testing with larger numbers of isolates to accurately determine susceptibility breakpoints for *M. haemolytica* 1:A. Although proteolytic enzymes in the bovine nasal mucosa may degrade the peptide and inhibit antibacterial activity, no inhibition was observed by incubating cecropin B in bovine nasal secretion. Furthermore, cecropin B was able to completely inhibit *M. haemolytica* 1:A growth in BHI broth within 20 minutes of incubation at 37°C.

M. haemolytica 1:A was not isolated from any calf in either experiment prior to challenge. After challenge, calves were stressed by transportation in a crowded trailer 100 miles for 3 hours. Seven out of the 8 control calves yielded detectible levels of *M. haemolytica* 1:A in nasal aspirates throughout the weeks following challenge. Calves aerosolized with 25 µg of plasmid DNA per nostril did not gain any protection to *M. haemolytica* 1:A. All 4 calves given 25 µg of plasmid DNA per nostril yielded detectible levels of *M. haemolytica* 1:A in nasal aspirates. Calves aerosolized with 50 or 100 µg of plasmid DNA per nostril appeared to gain some or total protection to *M. haemolytica* 1:A, respectively. Two of the 4 calves given 50 µg of plasmid DNA per nostril yielded detectible levels of *M. haemolytica* 1:A in nasal aspirates following challenge. However, *M. haemolytica* 1:A was not detected in any calf given 100 µg of plasmid DNA per nostril.

The percent frequency of nasal flora isolates were different between the summer (II) and fall (III) experiments, but were consistent between the groups in each experiment. These results indicate that these organisms were always present at some level, but may have been increased or reduced due to environmental temperature or competitive inhibition. There appeared to be no significant change in the normal bacterial nasal flora. However, there were drastic fluctuations in the average amounts of Gram-negative bacteria isolated from all groups in the fall experiment. Mean Gram-negative bacteria colony counts for calves given 50 µg or 100 µg of plasmid DNA per nostril dropped late during the experiment, after which no Gram-negative bacteria were isolated again. Mean Gram-negative bacteria colony counts for control calves also dropped late in the experiment, after which only small amounts of Gram-negative bacteria were detected. It would appear that transfecting the nasal passages of cattle may have affected the Gram-negative nasal flora. However, due to drastic fluctuations in the average amounts of Gram-negative bacteria isolated from all groups in the fall experiment, including the control calves, transfecting the nasal passages of cattle with a gene coding for cecropin B was not likely responsible for altering the normal nasal flora. While transfecting the nasal passage of calves with 50 or 100 µg of plasmid DNA per nostril did appear to inhibit colonization of *M. haemolytica* 1:A, it did not appear to alter the normal indigenous and transient flora.

It is beneficial to find new strategies for the control of pneumonic pasteurellosis. The use of gene therapy will help to eliminate primary *M. haemolytica* 1:A infection in cattle destined for the feedlots. The findings here indicate that antimicrobial peptides such as cecropin B are useful in inhibiting *M. haemolytica* 1:A colonization. They also

indicate that the upper respiratory tract may be transfected with genes coding for these antimicrobial peptides. Transfected calves expressing cecropin B in the nasal passage exhibited elevated resistance to *M. haemolytica* 1:A. This work will add important facts to the database necessary to plan strategies for control of shipping fever.

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APPENDIX A: LETTER OF PERMISSION

Louisiana Cattlemen's Association

4921 I-10 FRONTAGE ROAD
PORT ALLEN, LOUISIANA 70767-4195
TELEPHONE 225-343-3491 FAX 225-336-0002

EAT MORE BEEF

26 January 2004

Charles M. Boudreaux
Department of Veterinary Science
Louisiana State University
Baton Rouge, La. 70803

Dear Mr. Boudreaux,

Thank you for your correspondence of January 22nd requesting copies of the November 2003, issue of ***The Louisiana Cattleman*** magazine in which your article appeared.

I am enclosing 14 copies of this issue.

We hereby grant you permission to use your article "A novel strategy of controlling shipping fever" printed in the November 2003 issue of ***The Louisiana Cattleman*** as an appendix or chapter in your MS thesis.

Sincerely,

Sharon L. Hoffeld, Editor
The Louisiana Cattleman

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Enclosures

AFFILIATED WITH
National Cattlemen's Beef Association

APPENDIX B: A NOVEL STRATEGY OF CONTROLLING SHIPPING FEVER*

Bovine pneumonic pasteurellosis or shipping fever continues to be the major cause of clinical disease and death in feedlot cattle and has been estimated to result in over a billion dollars in annual losses in the beef cattle industry. Feeder calves from the southeastern United States normally break with the disease within 30 days after shipment. The bacteria *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) is the organism most commonly associated with the disease. There have been numerous attempts to develop effective vaccines against this disease since the 1950s, but despite these efforts, a vaccine that provides complete and consistent protection from pneumonic pasteurellosis in cattle is not available. Most veterinarians rely on antimicrobial agents to treat the disease. Streptomycin, tetracyclines, β-lactin antibiotics, chloramphenicol, sulfonamides, and trimethoprim-sulfonamide combinations have all been used to treat pneumonic pasteurellosis. Ironically studies have shown *M. haemolytica* to have an increasing pattern of resistance to all of these antimicrobial agents.

We believe that stressed cattle experience a multiplication of *M. haemolytica* bacteria in the upper respiratory tract. Stress usually comes in the form of transportation, but can include crowding, irregular feeding or watering, abrupt climate changes and exposure to viral agents or a combination of the above. Multiplication of *M. haemolytica* in the upper respiratory tract, especially the nasal cavity, results in deposition of the bacteria in the lung. This direct lung exposure results in both an immune and an inflammatory response that interferes with clearance of the bacteria from the lung and therefore causes pneumonia.

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* printed in the November 2003 issue of *The Louisiana Cattleman*

In the past we have shown that an aerosol vaccination of the upper respiratory tract resulted in deminished bacterial colonization of *M. haemolytica* in that area, as well as prevention of colonization in the lung. Rapid clearance of *M. haemolytica* was achieved by stimulating a specific class of secretory antibodies that act at the level of the mucosal surface of the nasal cavity. The importance of rapid clearance from the nasal cavity is that there is no longer a source of the organism for continued inhalation into the lung.

The device used to aerosolize the vaccine into the nasal passage was a siphon feed gun from a sandblasting system. The gun uses the flow of compressed air to create a siphon. We modified the gun in order to secure a tube of inoculum to the intake stem. We also attached a 3-inch piece of rubber tubing to the nozzle of the gun as an extension to ensure the vaccine was delivered into the nasal passage. A compressed nitrogen cylinder equipped with a regulator provided the 40-psi needed to aerosolize the vaccine.

Recently, this system was used to deliver a gene encoding an antibacterial protein called cecropin B. Cecropins were discovered in 1980, when it was noticed that the Cecropia moth (*Hyalophora cecropia*) responds to bacterial infection with very effective antibacterial activity. The pupae synthesize several different classes of antibacterial proteins, but the more potent of these are the cecropins. Cecropins are small strongly basic proteins, which have a broad spectrum of bactericidal activity, but are unable to disrupt mammalian cells. This clearly implicates their possible usefulness as novel antibiotics.

The cecropin B gene was first incorporated into a DNA vector which also contained genes encoding for all the enzymes necessary to transfer the cecropin B gene

into the recipient's DNA. The plasmid was then complexed to a lipid based transfecting reagent designed to deliver DNA to the nucleus of mammalian cells. The reagent complexes with DNA and arranges it in compact structures, to ease entry into the cell. This reagent also stabilizes the plasmid DNA inside the cell by protecting it from enzymes meant to destroy foreign DNA. The delivery system for the aerosol was the modified siphon-feed gun previously described. All personnel involved in the DNA delivery were protected by wearing disposable coveralls, disposable latex gloves and HEPA filtered respirators to prevent accidental exposure. The disposable coveralls and latex gloves were properly disposed as biohazardous waste.

Twenty 300-500 lbs. crossbred beef calves were brought to the LSU Veterinary Science Farm in Baton Rouge LA and kept on pasture. Calves were randomly assigned to 5 groups with 4 calves in each group. Three groups were designated the experimental groups, and the other two were designated the control groups. The 5 groups remained isolated throughout the experiment. Each of the experimental calves were given incremented amounts of DNA suspended in 3 ml of sterile Phosphate buffered saline (pH 7.2) per nostril, aerosolized with 40-psi of pressure. Control calves were aerosolized first, without DNA, then allowed to return to the pasture. The experimental calves were aerosolized one at a time, with that calf being the only calf allowed in the chute at the time. All 20 (experimental and control) calves were challenged intranasally with a virulent strain of *M. haemolytica* suspended in 3 ml of sterile Phosphate buffered saline (pH 7.2) per nostril. Following the inoculation, all calves were trucked separately (by groups) 100 miles for 3 hours to stress them. Nasal swabs were collected on days 0, 3, 7,

14, 21 and 28. Samples were submitted for bacterial culture. Day 0 samples were collected prior to any treatment.

M. haemolytica was not isolated from any calf at day 0. However, after challenge *M. haemolytica* was isolated from 7 of 8 control calves at some point during the experiment. *M. haemolytica* was isolated from 4 of 8 control calves at day 3, and 5 of 8 calves from days 7 thru 28. *M. haemolytica* was isolated from all 4 calves given 25μg of DNA per nostril at day 3. *M. haemolytica* was also isolated from all 4 of these calves on days 7, 14, 21 or 28. *M. haemolytica* was isolated from only 1 of 4 calves given 50μg of DNA per nostril at day 3, and only 1 of 4 calves from days 7 thru 28. *M. haemolytica* was not isolated from any of the 4 calves given 100μg of DNA per nostril at any point of the experiment.

Table B.1. Summary of *Mannheimia haemolytica* isolated

GROUP	Day 0	Day 3	Day 7-28	TOTAL
Controls	0 of 4	4 of 8	5 of 8	7 of 8
E25	0 of 4	4 of 4	4 of 4	4 of 4
E50	0 of 4	1 of 4	1 of 4	2 of 4
E100	0 of 4	0 of 4	0 of 4	0 of 4

E25, Calves given 25 ug/ml of DNA per nostril
E50, Calves given 50 ug/ml of DNA per nostril
E100, Calves given 100 ug/ml of DNA per nostril

It is beneficial to find novel strategies for the control of pneumonic pasteurellosis. The findings indicate that novel antimicrobial proteins such as cecropin B may be useful in inhibiting *M. haemolytica* colonization. They also indicate that the upper respiratory tract may be transfected with the genes coding for these novel antimicrobial proteins. Transfecting the nasal passage of calves with sufficient copies of the cecropin B gene was

shown to inhibit colonization of *M. haemolytica*. Inoculating calves with 100µg of plasmid DNA per nostril completely inhibited *M. haemolytica* colonization. Inoculating calves with 50µg of plasmid DNA per nostril provided some resistance to *M. haemolytica* when compared to control calves. However, inoculating calves with 25µg of plasmid DNA per nostril provided little or no resistance to *M. haemolytica* when compared to control calves. This work will add important facts to the database necessary to plan strategies for control of shipping fever.

VITA

Charles Mitchell Boudreaux is the son of Kenneth P. Boudreaux and Annabelle L. Boudreaux from New Iberia, Louisiana. He was born on March 18,1974, in New Iberia, Louisiana. He attended Avery Island Elementary for grades one through six, New Iberia Middle School for grades seven and eight, and graduated from New Iberia Senior High in 1992. While in high school he was an active member of the 4-H Club, participating in livestock exhibition, livestock judging, entomology, and leadership programs. In the spring of 1998 he graduated from Louisiana State University in Baton Rouge with a Bachelor's degree in animal sciences. Following graduation he took a position as a research associate in the Department of Veterinary Science with Dr. Richard Corstvet. In the fall of 2001 he began working on a master of science degree in Veterinary Medical Sciences under the direction of Dr. Richard Corstvet. Charles is now a candidate for his master of science degree in Veterinary Medical Sciences through the Department of Pathobiological Sciences, Louisiana State University, School of Veterinary Medicine.