DEVELOPMENT OF IN VITRO AND EX VIVO

MODELS FOR STUDY OF ADHERENCE

OF PASTEURELLA HAEMOLYTICA TO

BOVINE MUCOSAL TISSUES


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CHAPTER I

INTRODUCTION

Bovine respiratory disease (BRD) has long been a problem in stocker and feeder operations and is responsible for considerable economic loss to the US beef industry (USDA, 1995). Methods of raising and marketing cattle in this country promote compromisation of the calf's innate respiratory defense mechanisms, making these animals susceptible to infection by *Pasteurella haemolytica* serovar A1 (PhA1), the bacterium most frequently isolated from the lungs and nasal passages of cattle suffering from acute BRD (Frank, 1979 and 1984; Frank and Smith, 1983). The specific mechanism whereby PhA1 invades the lung has not been well established, but many field studies conducted over the last 25 years have indicated that pneumonia is preceded by preferential colonization of the upper respiratory tract (URT) by PhA1 (Frank, 1986).

*Pasteurella haemolytica* is isolated in low numbers from the nasal passages of healthy cattle. However, most of these isolates are *P. haemolytica* serovar A2 (PhA2) (Frank and Smith, 1983; Purdy *et al*, 1986), whereas those from cattle with signs of BRD are predominantly PhA1, and these in high numbers (Frank *et al*, 1986; Frank *et al*, 1987; Frank *et al*, 1994). It has recently been shown that the most likely source of PhA1 is the healthy calf's palatine tonsils which act as a reservoir for low numbers of PhA1 (Frank *et al*, 1986; Frank and Briggs, 1992). In order for the establishment of PhA1 infection in the nasopharynx, the calf's respiratory tissue must be in a condition conducive to bacterial replication. This condition is apparent when a respiratory viral infection and/or stress,

particularly that of transportation with overcrowding, compromise the URT defense mechanisms (Frank, 1986). There follows a rapid multiplication of PhA1 in the nasopharyngeal area, and it is suspected that not only are these bacteria horizontally transmitted from the nasopharynx of one stressed or viral-infected calf to another (Briggs *et al*, 1998), but that a large number of PhA1 are inhaled as aerosolized droplets into the lungs, where they continue to replicate causing a fibrinopurulent bronchopneumonia (Jericho *et al*, 1986; Frank *et al*, 1986; Whiteley *et al*, 1992).

Generally, for rapid colonization of the URT to occur, pathogenic bacteria must adhere to the host cell surface (Beachey, 1981). The ability of PhA1 to preferentially multiply and colonize the nasopharyngeal epithelium of stressed and/or viral-infected cattle may be due to its ability to adhere to the URT mucosa. A review of current literature reveals that studies have yet to be conducted to show whether *P. haemolytica* actually adheres to URT tissue, and if so, exactly where or how this adherence takes place. Outer membrane proteins, capsule, or putative fimbriae are the most likely contenders for bacterial adhesins, but neither these nor the host cell receptors, be they on cilia, epithelial cell surfaces, or mucus, have been identified.

Lack of published studies on adherence of PhA1 to bovine URT tissue is in part due to the difficulty in obtaining suitable bovine models. The logistics and expense of working with cattle and the complexity of the natural URT environment severely limit *in vivo* studies of certain isolated host and bacterial factors critical to early pathogenesis. Thus, available *in vitro* models i. e., intact excised tissue (*ex vivo*) (Read *et al*, 1991), tissue culture (St. Geme and Falkow, 1990), or scraped epithelial cells (St. Geme and Cutter, 1996), must be adapted for use, or new models developed, to study the adherence

of PhA1 to bovine URT tissue. The development of appropriate models will aid in elucidation of conditions necessary for, and bacterial and host factors involved in, adherence of PhA1 to mucosal cells.

Thus, the overall goal of this research is to develop appropriate models for the study of PhA1 adherence to bovine URT tissue. It is expected that the availability of these models will facilitate investigation of the early pathogenesis of pneumonic pasteurellosis and development of effective strategies for management of the disease.

CHAPTER II

LITERATURE REVIEW

Bovine respiratory disease (BRD), also known as bovine pneumonic pasteurellosis or shipping fever, continues to be of considerable economic importance to the beef industry worldwide. Death, decreased animal performance and productivity, as well as medical and labor costs, have a tremendous economic impact, particularly on stocker and feeder operations. Despite many improvements in herd health and management practices in the USA over the last decade, the percentage of cattle/calf deaths (estimated at 31.1 % in 1992) and the economic loss due to respiratory disease continues to rise (estimated at near $600 million in 1992 to near $1 billion in 1997) (USDA, 1995; Griffin, 1997), indicating that much work still needs to be done to gain a better understanding of this disease complex and its therapeutic management and prevention.

Etiology

Acute BRD has been investigated since the late 1950s, but only in the early 1980s after years of clinical case studies and accumulation of observations, was it realized that BRD is not caused by a sole etiologic agent but by the interaction of a number of factors, including viruses, bacteria, and various stressors which compromise the innate respiratory defense mechanisms (Thomson, 1981).

Bacteria

The nasal flora of healthy, unstressed cattle consists of a wide variety of bacteria, which shift in the predominant species over time within an individual (Magwood *et al*, 1969) and even between individuals within a herd (Frank, 1984). *Pasteurella haemolytica* [recently renamed *Mannheimia haemolytica* for biotype A isolates (Angen *et al*, 1999), but shall be referred to as *Pasteurella haemolytica* throughout this dissertation] is found as part of this bacterial flora in the nasopharynx of healthy calves. However, as it usually comprises such a small percentage of the total flora, it is only sporadically detected by nasal swab cultures (Magwood *et al*, 1969; Frank and Smith, 1983).

Information on the serology of *Pasteurella haemolytica* became available in circa 1960 when serotyping by indirect haemagglutination (Biberstein *et al*, 1960) demonstrated 11 defined serological types. Smith (1961) further divided the species according to biotype, where *Pasteurella haemolytica* was differentiated on its ability to ferment either arabinose (A biotype) or trehalose (T biotype), each biotype being associated with distinct clinical syndromes. In cattle, 16 serotypes (serovars) of biotype A are now recognized (A1 – A16). However, a recent isolate from sheep in Syria has been identified as serovar A17 (Younan and Fodar, 1995), and may also prove to be present in cattle. Serovar A2 is the most frequent isolate from the nasal passages of healthy cattle (Purdy *et al*, 1986) and serovar A1 is the major isolate found in the nasal flora of cattle with acute BRD (Wray and Thompson, 1971; Fox *et al*, 1971; Frank, 1979 and 1984), and is almost exclusively isolated from pneumonic lungs in fatal cases of BRD (Thomson *et al*, 1969; Wray and Thompson, 1971; Fox *et al*, 1971; Reggiardo, 1979; Allan *et al*, 1985; Binkhorst *et al*, 1990; Purdy *et al*, 1997).

*Pasteurella* species are not thought to be inhabitants of the lower respiratory tract of healthy cattle. No Pasteurellaceae have been isolated from the lungs, bronchial lymph nodes or tracheas of healthy cattle at slaughter (Collier and Rossow, 1964; Yates *et al*, 1983), although it has been recorded that *P. haemolytica* and *P. multocida* were isolated in small numbers from nonpneumonic lungs of housed calves less than 6 months of age (Allan, 1978) and that *P. haemolytica* was isolated from the tracheal air of calves carrying detectable numbers of these bacteria in their nasal passages (Grey and Thomson, 1971).

*Pasteurella multocida* is another common isolate, but it is usually found in younger (<6 months) cattle (Griffin, 1993), and in chronic cases of BRD. *Pasteurella multocida* Carter's Type A (Carter, 1973) and Heddleston's Type 3 (Blackburn *et al*, 1975) are the most commonly found *P. multocida* isolates.

*Haemophilus somnus*, which causes several disease syndromes, including septicemia, thromboembolic meningoencephalomyelitis, and fibrinous bronchopneumonia in cattle, is commonly isolated from pneumonic lungs of yearling cattle in Canada (Harris and Janzen, 1989). *Arcanobacterium pyogenes* can also be isolated, but usually from chronic cases of BRD. *Mycoplasma bovirhinis*, *M. bovis*, *M. dispar*, and *Ureaplasma* spp. (Ernø, 1978), as well as *Chlamydia* spp. have been isolated in association with other respiratory bacterial pathogens and their contribution to BRD is unclear (Thomson, 1981).

Viruses

Although bacterial pneumonia, with *P. haemolytica* A1 as the key agent, appears to be the cause of death in most cases of acute BRD, the role of viruses in this disease

complex cannot be underestimated. Respiratory viruses clearly modulate host-defense mechanisms and alter the URT and lung environment, such that bacterial superinfection can occur. Bovine pneumonic pasteurellosis is thought to be caused by the interaction of stressors and infection by viruses and bacteria acting synergistically (Babiuk *et al*, 1995). Certain studies conducted in experimentally infected calves have shown that animals carrying PhA1 in very low numbers in the nasopharynx, can become active shedders when developing viral respiratory tract disease (Frank *et al*, 1986), and calves that are not carriers of PhA1 but have viral respiratory tract disease are highly susceptible to PhA1 colonization, and subsequently become active shedders from the nasal passages (Frank *et al*, 1987).

There are three important viruses that directly affect the bovine respiratory tract and that have long been established as important viral components in the BRD complex. Bovine herpes virus 1 or infectious bovine rhinotracheitis virus (IBRV) is a commonly found respiratory virus that usually affects cattle older than 6 months. Respiratory infection varies in severity depending on the strain involved, and the symptoms can range from inapparent/mild signs to severe disease (usually as a result of secondary bacterial pneumonia) with about a 10% mortality in severe outbreaks in feedlots (Kapil and Basaraba, 1997). Infection with IBRV causes a necrosis of the epithelium of the URT that compromises mucociliary clearance of *Pasteurella haemolytica*, leading to increased colonization and multiplication, and ultimately bronchopneumonia. Bovine parainfluenza 3 virus (PI3V) is the most consistent viral isolate in cases of BRD. Alone, this ubiquitous paramyxovirus causes only mild clinical signs in young calves (2 – 8 months) and occasionally yearling cattle (Bryson *et al*, 1979), infecting ciliated

respiratory epithelium of the URT as well as alveolar epithelium and macrophages. Its importance in BRD, as with IBRV, appears to be predisposition to secondary bacterial pneumonia due to viral-mediated alterations in bactericidal ability of macrophages, decreased bacterial clearance, and immunosuppression. The paramyxovirus, bovine respiratory syncytial virus (BRSV), commonly infects young healthy cattle causing an asymptomatic or mild acute respiratory disease, although it is known to result in severe clinical signs of bronchointerstitial pneumonia in newly weaned calves less than 6 months of age (Baker, 1993). This virus infects and causes necrosis of several ciliated and nonciliated epithelial cell types in the respiratory tract, resulting in necrotizing bronchiolitis that is characteristic of severe BRSV infections. As with IBRV and PI3V, there appears to be a strong correlation between BRSV infection and BRD (Baker et al, 1986; Stott et al, 1980).

Bovine viral diarrhea virus (BVDV) infections in cattle may result in one of three disease syndromes: bovine viral diarrhea, mucosal disease, and fetal disease. Respiratory tract infections due to opportunistic bacteria may occur in unthrifty, persistently-infected calves or in cattle suffering from generalized bovine viral diarrhea. In either case there is impairment of peripheral immunity and pulmonary resistance (Al-Darraji et al, 1982). However, the degree to which BVDV contributes to the incidence of BRD is a contentious issue among researchers. BVDV can be recovered with high frequency from pneumonic lungs of cattle (Reggiardo, 1979) and infections with this virus are often associated with BRD in calves (Stott et al, 1980). Initial replication of the virus occurs in the respiratory tract and tonsils (Bolin, 1992), and it is believed that certain strains of this virus have the capacity for producing a mild respiratory tract disease. However, there is

little direct experimental evidence that establishes BVDV as a primary pathogen causing pneumonia. Thus, its primary role in BRD may be its capacity to facilitate and enhance secondary infections. Experimental evidence also supports the conclusion that BVDV enhances the incidence and severity of respiratory tract disease caused by IBRV, BRSV, or *P. haemolytica* (Potgieter, 1997).

Bovine respiratory coronavirus (BRCV) causes upper and occasionally lower respiratory tract disease in adult cattle, especially after shipping to feedlots (Storz *et al*, 1996; Heckert *et al*, 1989). The respiratory isolates are related antigenically to the enteric isolates of bovine coronavirus, which are known to establish chronic infections in the intestinal tract of calves and adult cattle (Zhang *et al*, 1994). Based on experimental infections, calves with BRCV-associated respiratory disease develop interstitial pneumonia, but whether the virus predisposes cattle to bacterial infection in BRD is under investigation (Kapil and Goyal, 1995).

Adenovirus and rhinovirus have also been associated with acute BRD (Smith, 1984).

Stressors

Healthy, unstressed cattle are not readily colonized by experimental aerosol or intranasal inoculation of *Pasteurella haemolytica* normally isolated from bovine pneumonic lungs (Frank, 1986), unless the respiratory immunity is compromised by viral infection or other stressors. Frank and Smith (1983) noted that the frequency of *P. haemolytica* A1 isolations increased dramatically when cattle were shipped from farm to feedlot, indicating that stressors, particularly those of transportation and crowding, seem to promote the development of BRD.

There is very little information on how stress in cattle alters and allows colonization of the URT by bacteria. In stressed human patients after surgery, there is an increased salivary protease production, decreased fibronectin on URT epithelial surfaces, and increased susceptibility to colonization by gram-negative bacteria. Thus it appears that protease alters the amount of fibronectin, which in turn, exposes epithelial cell surface receptors permitting adherence of gram-negative bacteria (Woods, 1987; Johanson *et al*, 1980). The effect of stress on mucus is also unclear. However, adrenergic stimulation of respiratory mucus glands increases the fluidity of secretions in most animal species. This in turn, decreases the effective movement of mucus by cilia (Marin, 1986).

The emergence of common bacterial pneumonias appears to have parallelled the development of modern management and marketing systems. In the US, newly-weaned beef calves are often brought together from a number of different sources, held, and then transported long distances to sale barns and /or feedyards. In Europe and the U. K., there is intensive raising of housed dairy calves. These stressful situations, often exacerbated by environmental factors such as heat, cold, rain, dust, or toxic fumes, and dehydration during transportation, set the stage for development of acute BRD.

Studies on whether nutritional factors play a role as stressors are inconclusive, but copper and vitamin E deficiencies have been implicated (Smith, 1984; Thomson, 1981).

Heredity

The relationship between BRD and heredity is unclear, although there is a suggestion that this may exist. In experiments conducted on cattle in feedlots, certain cattle breeds, such as Pinzgauer, Hereford, and Simmental demonstrated a higher BRD

frequency, but heritability estimates of BRD during pre- and post-weaning periods did not differ significantly from 0, indicating that selection for resistance to BRD is slow when using disease occurrence as the only selection criterion (Muggli-Cockett *et al*, 1992).

### Epidemiology and Pathogenesis of Upper Respiratory Tract Infection

Almost all knowledge to date about colonization of the bovine upper respiratory tract by *P. haemolytica* is based on *in vivo* studies, the majority of which have been conducted by Frank (1988), Frank and Briggs (1992), and Frank *et al* (1986, 1987, 1989, 1994). Although *in vivo* studies cannot be used to ascertain specific interactions at the cellular and molecular level, the many field studies conducted and the number of observations made over the 25 years of study of clinical cases, have been invaluable in determining a number of facts pertaining to the early pathogenesis of BRD.

*Pasteurella haemolytica* is thought to be a commensal of the nasopharynx of healthy calves. However, these bacteria are only found sporadically and in low numbers in the nasopharynx. In an early study, a large number of healthy calves sampled by weekly nasal swabs over seven months, produced very few *P. haemolytica* isolations (Magwood *et al*, 1969). A later field observation confirmed that most healthy, non-stressed cattle at the farm of origin carried *P. haemolytica* in the nasal passages in very low numbers, and even at undetectable levels for extended periods of time (Frank and Smith, 1983).

It was assumed from early surveys that the *P. haemolytica* sporadically found in nasal passages of healthy calves were the same bacteria that caused BRD. However, after development of serotyping and upon closer investigation, it was shown that most isolates from healthy animals were *P. haemolytica* A2 (Frank and Smith, 1983; Purdy *et al*, 1986), whereas those from cattle with signs of respiratory disease were usually *P. haemolytica* A1 (Frank, 1984). *P. haemolytica* A1 is very rarely isolated from healthy cattle.

It was also assumed until recently that the healthy calf's nasopharynx acted as a reservoir for PhA1. It is true that in recently shipped and virally-infected calves, the immediate source of PhA1 that causes pneumonic pasteurellosis in a calf is its own heavily colonized nasopharynx. However, so few PhA1 can be detected in the nasopharynx of healthy calves that colonization is probably initiated either by horizontal aerosol transmission from the nasopharynx of another calf (Briggs *et al*, 1998) and/or from a site distinct from the main nasal passages, i.e. the tonsils (Frank and Briggs, 1992). When *P. haemolytica* was instilled into the tonsillar sinuses of ten healthy calves, bacteria were detected in tonsillar wash for 3 weeks after instillation in the absence of clinical signs (there was a serum and nasal secretion antibody response), indicating that *P. haemolytica* can be carried at this site in healthy calves. However, *P. haemolytica* was detected in the nasal mucus of only 2 calves for one week post-inoculation, and all other calves cultured negative, confirming that the tonsils were not continually shedding large numbers of bacteria in the nasal mucus, and that which was shed, was rapidly cleared (Frank and Briggs, 1992). Thus, the tonsils appear to be a likely reservoir for PhA1.

The exact location of *P. haemolytica* in the nasopharynx of healthy calves that are carrying small numbers of the organism is unknown (Thomson *et al*, 1969). However, early studies of recently shipped calves have shown that areas of the nasal passage from which the organism can be isolated are the lower nasal cavity and lateral anterior turbinate. When *P. haemolytica* replicates to very high numbers it can then be isolated from all areas of the nasal passages (Pass and Thomson, 1971). Attempts to establish long-term nasal colonization of PhA1 in healthy, unstressed calves by instillation of a broth culture or aerosol exposure of PhA1 into the ventral nasal meatus resulted in the inoculum being rapidly cleared from the nasopharynx, and in half the cases PhA1 was not detectable for more than 1 day (Frank *et al*, 1986). This lack of success was probably due to the good mucus flow and competition with indigenous bacterial flora in the nasal area. Despite clearance from the nasopharynx, there was a serum and nasal secretion antibody response. In contrast, the middle nasal meatus has a reduced mucus flow and bacterial flora, and has been shown to retain instilled PhA1 longer because of its anatomical structure, resulting in PhA1 being detectable for an average of 7 days post-exposure. Serum and nasal secretion antibody concentrations were also increased. However, when these same calves were exposed to IBR virus four weeks later, only half were induced to shed PhA1, while the rest did not shed as they were probably no longer harboring the organism (Frank *et al*, 1989).

In order for colonization to occur, calves must be in a physical condition conducive to rapid multiplication of *P. haemolytica* to high numbers on the susceptible nasopharyngeal surface. These conditions appear to be present when there is crowding of calves which promotes the spread of respiratory viruses and *P. haemolytica*, when calves

are stressed by such events as transportation, inclement weather, and/or weaning, and when the health of calves is impaired, usually by respiratory viruses. Studies have been undertaken that involved exposing calves that had previously been infected with IBR virus to nasal instillation of PhA1. These calves were readily colonized by PhA1, which was shed for more than 1 day in all calves, and in three-quarters of the cases was shed for greater than 7 days (Frank *et al*, 1987). In another study, calves that had been exposed to PhA1 and then nasal-cultured negative for PhA1 for several weeks, were infected with IBR and PI-3 viruses. These cattle shed detectable numbers of *P. haemolytica* in nasal secretions during the viral clinical illness, indicating that the nasal and serum antibodies produced in calves previously exposed to PhA1 via the respiratory tract were not protective against PhA1 during severe viral respiratory disease (Frank, 1986). Another study to look at the effect of climate on clearance of the organism from the lung, revealed that a change from a cold and dry climate to a hot and humid one, caused *P.haemolytica* to rapidly colonize the nasopharynx (Jones, 1987).

Virus infection has been shown to influence adherence and colonization of bacteria by altering the host cell surface membrane receptors and the micro-environment where bacterial attachment occurs (Babiuk *et al*, 1995). Viral damage and necrosis of the URT epithelium, causing loss of cilia or alteration of mucus and ciliated cell function, compromises mucociliary clearance of bacteria. This in turn, may lead to increased colonization and bacterial growth. Viruses also induce the production of cytokines which directly or indirectly alter macrophage, polymorphonuclear neutrophil (PMN), and lymphocyte activity, which play a role in clearing bacteria from the LRT.

Divalent cations such as zinc and iron are also increased following URT infection with viruses and these may influence adherence of bacteria, production of adhesins, and immune responses. In many cases, iron is an essential element for bacterial growth, and iron-regulated outer membrane proteins are involved in modulating virulence. Also, iron bound to transferrin has been shown to be critical in immune regulation. Surfactant and fibronectin production can also be affected. Proteases produced by viral-infected cells or bacteria not only cleave fibronectin, exposing host receptors and increasing the ability of bacteria to adhere, but they cleave viral glycoproteins enhancing viral infectivity, and inactivate secretory immunoglobulin.

Thus, colonization of the nasopharynx appears to be triggered even in the face of immunity, provided there is some alteration in bacterial, host, and/or environmental factor. When one or more of these conditions are present, there follows a rapid multiplication of PhA1 in the nasopharyngeal area, which supplies the critical numbers of bacteria to repeatedly expose the lung via aerosolized droplets (Grey and Thomson, 1971; Jericho *et al*, 1986), resulting in pneumonia.

Bacterial Adherence

General

Koch's postulates were originally developed for identifying epidemic bacterial diseases and were certainly an important basis for the scientific study of these diseases in the 1800s. However, the implication that virulence is a characteristic of the bacterium and is independent of the host, and the assumption that all bacteria of one species are

equally virulent, is now clearly erroneous. The ability of a bacterium to cause infection, i.e. its virulence, depends on bacterial products and/or strategies which may vary between strains of the same bacterial species, and on the susceptibility of the host.

Once bacteria have overcome the many host protective mechanisms preventing microbial entry, the first major interaction between pathogenic bacteria and their host usually involves attachment to eukaryotic cell surfaces (Beachey, 1981). Here the bacteria can either multiply and remain on the cell surface, or they can use this attachment as a step to deeper tissue invasion and possibly intracellular uptake, or to relocate to a different area (Ofek and Beachey, 1980a). Thus, blocking initial attachment of the pathogenic organism would be an important means of preventing establishment of infection.

Bacterial attachment or adhesion prevents the pathogen from being eliminated by the host defense cleansing mechanisms on endothelial and mucosal cell surfaces (Ofek and Beachey, 1980b; Beachey, 1981). Unattached bacteria are easily swept away by such cleansing mechanisms as sneezing, coughing, mucociliary clearance, and blood flow. Those that attach must constantly multiply to avoid removal during normal cell desquamation. This adherence not only allows bacterial multiplication, but enables bacteria to better resist deleterious agents, competition from resident microflora, and local immune mechanisms, and enhances toxicity to the host (Ifek and Doyle, 1994).

Microbial adherence requires recognition of complementary surfaces on both the bacterium and the eukaryotic cell. Simply, this recognition involves the interaction between receptors on or near the eukaryotic cell surface, and adhesins on the bacterial cell surface. Bacterial adhesins are typically protein structures on the bacterial surface,

and these engage in protein-carbohydrate or protein-protein interactions with host cell glycolipids (Karlsson, 1989) or integral membrane glycoproteins (Ruoslahti, 1991), or with matrix glycoproteins such as fibronectin found in extracellular matrices and blood (Ruoslahti, 1988). A few eukaryotic interactions have suggested carbohydrate-carbohydrate binding (Turley and Roth, 1980), but this type of bacterial adherence has not yet been observed, except to host mucus (Uhlich *et al*, 1993).

Microbial adherence, although well studied for many pathogenic bacteria (Knutton *et al* 1984; Reid and Sobel, 1987), is difficult to define in terms of adhesin contribution to tissue colonization. For example, *Escherichia coli*, which bears mannose-specific adhesins, does not colonize all mannose-containing tissue (Ifek and Doyle, 1994). This phenomenon suggests that the adhesin and the host receptor probably need to be correctly presented, orientated, and reasonably accessible. Furthermore, certain bacteria express several alternative means of cell attachment, yet some of these may only be expressed under certain environmental or host conditions. It is conceivable that any bacterial surface antigen which has a stereochemical conformation, hydrophobicity profile, and net electrostatic charge, complementary with membrane structures on a target cell, could evolve as a potential adhesin under selective pressures of the host environment. In fact, many pathogens show phase variation, that is, are capable of expressing or repressing expression of adhesins, depending on a number of factors such as stage of growth, nutritional status, temperature, pH, and type of media. This phase variation may allow the pathogen to initially adhere to the epithelial surface and establish colonization, but to dissociate at the time of tissue invasion and actual infection, thereby evading interaction with phagocytes. Alternatively, adhesins may act together to increase

the strength and specificity of adherence, as occurs with pertussis toxin and filamentous haemagglutinin of *Bordetella pertussis* (Sandros and Tuomanen, 1993; Tuomanen *et al*, 1986). In addition, there may also be a species specificity, that is, a correlation between host susceptibility and ability of eukaryotic cells to bind the pathogen. This occurs with many pathogens, including *Bordetella pertussis* which adheres well to human ciliated cells but not to the same cells of other mammalian species (Tuomanen *et al*, 1983). Certain pathogenic bacteria also have the ability to differentiate target tissues within the host, that is, show tissue tropism. For example, *Bordetella pertussis* adheres exclusively to human lung ciliated epithelium and is non-invasive (Tuomanen, 1988). In contrast, *Streptococcus pneumoniae*, which also targets human lung tissue as an alveolar pathogen, can spread systemically to infect the middle ear and brain (Burman *et al*, 1985).

## Bacterial Virulence Factors involved in Adherence

### a) Fimbriae/pili

Bacteria employ a number of mechanisms through which they attach to host tissues. One of the better studied mechanisms involves the use of rod-shaped protein structures called fimbriae (or pili). The common pili or type I fimbriae are composed of a major protein made up of a group of identical repeating subunits with minor proteins at the tip or occasionally inserted along the length of the fimbriae. These are found on many members of the *Enterobacteriaceae*. In many cases, binding of fimbriae to the host cell receptor, eg, D-mannose (Eisenstein, 1988), has been shown to be mediated by a minor protein and not the major pilin structural unit (Minion *et al*, 1986). The major protein exhibits much variation among different enteric species, yet the minor tip adhesin

is conserved in many of the type I fimbriated *Enterobacteriaceae* species (Abraham *et al*, 1988).

The question of why many bacteria need long, fragile structures for adherence is not clear. The reason for the fragility of the pili, which are constantly being lost and reformed by bacteria, especially urogenital tract bacteria, would perhaps be a way to evade the host's immune response. A constant change in pilin type would make it difficult for pilus tip antibodies to block adherence. The reason for the length of the pili could be that bacteria and most biological tissues are thought to be negatively charged, thus allowing pili contact with receptors on tissue without getting close enough for electrostatic repulsion to prevent adherence (Ifek and Doyle, 1994). Yet this cannot be the complete explanation, as many bacteria are capable of a tight form of binding. Possibly the pili allow initial loose contact, so that other bacterial surface proteins can then bind more closely.

This phenomenon has been shown by St. Geme and Cutter (1995) who identified two morphologically distinct filamentous adhesive structures on the surface of *Haemophilus influenzae* type b strains. These structures appear to have separate cellular binding specificities for cultured epithelial cells. The researchers noted that, although haemagglutinating pili (approximately 5 nm diameter and 450 nm in length) were involved in adherence to human oropharyngeal cells (Guerina *et al*, 1982), adherence to human nasopharyngeal organ culture (Farley *et al*, 1986), cultured human conjunctival cells (St. Geme and Falkow, 1991), and Chang, HeLa, and Hep-2 epithelial cells (St. Geme and Cutter, 1996) was independent of haemagglutinating pili, suggesting a second adhesin. This was also suggested in a monkey-colonization study (Weber *et al*, 1991),

where non-piliated bacteria retained the ability to colonize (although to a lesser degree than piliated bacteria), and almost all bacteria recovered from the nasopharynx after original infection with the piliated strain, were observably non-piliated. Using transmission electron microscopy (TEM), St. Geme and Cutter (1995) observed small, thin, surface fibrils (2 nm diameter, 50–100 nm in length), and thereafter located a genetic locus involved in expression of these fibrils for adherence.

The existence of fimbrial structures on *P. haemolytica in vivo* and *in vitro* have been reported. Two types of fimbriae on *P. haemolytica* A1 grown *in vitro* were demonstrated by Morck *et al* in 1987. A large, 12 nm diameter, rigid structure, and a smaller, 5 nm diameter, flexible structure were seen using TEM. Pulmonary lavage fluid recovered from an experimentally infected calf also revealed fimbriae of about 10nm diameter on *P. haemolytica* (Morck *et al*, 1988). As well as this, linear structures suggestive of fimbriae were seen on *P. haemolytica* adherent to tracheal epithelium in a naturally infected calf (Morck *et al*, 1989). Also at this time, Potter *et al* (1988), purified the larger, rigid fimbriae by mechanical shearing and centrifugation, and characterized them using SDS-polyacrylamide gel electrophoresis and immunoblotting. These were found to be 12 nm in diameter with a molecular weight of 35000. All further electron microscopic studies using similar procedures have failed to demonstrate fimbriae on the surface of *P. haemolytica* (Richards A, unpublished data, 1989; Murphy G, unpublished data, 1992; Gonzalez and Maheswaran, 1993). However, it has been suggested that mechanical factors such as agitation (Morck *et al*, 1987), or phase variation of fimbriae expression (Mason, 1993) could prevent demonstration of fimbriae in various laboratories. Although recent findings in many studies of bacteria involved in respiratory

disease do indicate that fimbriae are important in upper respiratory tract colonization, until well-defined fimbrial structures are confirmed on *P. haemolytica*, it must be assumed that other surface components may function as adhesins.

b) <u>Capsule</u>

The capsule (or glycocalyx) of bacteria are in most cases, composed of highly hydrated polysaccharides, which are arranged as a relatively unstructured network of fibers emerging more or less vertically from the plane of the outer membrane, and usually extending out further than the O antigens of the lipopolysaccharide (LPS) (Bayer and Bayer, 1994). Capsules provide bacteria with a selective advantage over those that are noncapsulated, and serve a number of protective functions, as well as possibly playing a role in adherence to mucus or epithelial cell surfaces.

The main function of the capsule is to protect the bacteria from the host's inflammatory response, including complement activation and phagocyte-mediated killing, allowing them to survive *in vivo* prior to the development of specific antibody. For complement activation in the alternative pathway, serum C3b and Bb proteins must bind on the bacterial surface, to form the enzyme, C3 convertase. Some capsules prevent the formation of C3 convertase by failing to bind serum protein B, a prerequisite for this enzyme. Yet other capsules, such as those containing sialic acid, have a higher affinity for serum protein H, which binds to C3b allowing degradation by another serum protein, I, which inhibits the cascade. Some bacteria, such as *Haemophilus influenzae*, have O side-chains with an increased length beyond the capsule, which hold the formed C3 convertase away from the membrane, and thus prevent membrane attack. But not all

encapsulated bacteria automatically become serum resistant, as proteins can still diffuse through a loose capsule network to the membrane. Although serum-resistance can be attributed to the capsule of *P. haemolytica*, antibodies have been shown to be bacteriocidal through both the alternative and classical complement pathway (McVey and Loan, 1989).

By preventing C3 convertase formation, the capsule also decreases opsonization as C3b is not available for recognition by macrophages which have C3b receptors on their surface. There is also a decrease in the production of serum protein C5a which is responsible for chemotaxis. These in turn, decrease phagocyte-mediated killing. The capsule also helps protect the cell from certain antibiotics, bacteriocins, attack by bacteriophages, and from dessication, possibly increasing survival in the environment and thus transmission to a new host.

Capsules are known to be very poor immunogens, especially those with sialic acid or hyaluronic acid residues, which have shared antigenicity with similar residues commonly found in mammalian tissue, and to which the host is usually self-tolerant (Moreno *et al*, 1985). Many of the more pathogenic species in the family *Pasteurellaceae*, including *P. haemolytica*, are encapsulated, the capsule being produced during the logarithmic phase of growth. Those capsules that have been purified and analyzed have been found to be acidic, negatively-charged, high molecular weight polysaccharides, composed of repeating units of two or three sugars or aminosugars. The capsule of each of 5 different serovars of *P. haemolytica* (A1, A2, A7, T4, and T15), have been shown to have different sugar compositions (Adlam *et al*, 1986). Of the two most commonly found serovars, the capsule of *P. haemolytica* A1 is a mannopyranosyluronic

acid derivative [-3)-β-N-acetylaminomannuronic-(1-4)-β-N-acetylmannosamine-(1-(1,2)] (Adlam *et al*, 1984; Brogden *et al*, 1989) which is similar to that of certain enterobacteria. Antibodies to serovar A1 capsular polysaccharide are evoked following intradermal vaccination of calves with live organisms or capsular polysaccharides in oil adjuvant (Adlam, 1989). The capsule of *P. haemolytica* A2 is colaminic acid (also known as a sialic or acetylneuraminic acid derivative (2,8α-linked acetylmannosamine and pyruvic acid) (Adlam *et al*, 1987). The latter capsule is identical to that of *Neisseria meningitidis* group B and *E. coli* K1, both of which cause meningitis in infants (Moreno *et al*, 1985). In fact, monoclonal antibodies raised against *N. meningitidis* group B capsule gave a strong indirect hemmaglutination reaction with *P. haemolytica* A2, and were protective against septicemic A2 infection in mice, indicating that antibodies to A2 capsule may be important in immunity (Moreno *et al*, 1983), despite the fact that A2 capsule does not evoke a good immune response probably because it contains sialic acid (Adlam, 1989). The capsule of *P. haemolytica* can be seen by electron microscope when the organism is grown in culture (Corstvet *et al*, 1982; Morck *et al*, 1987; Gilmour *et al*, 1985) or in bovine subcutaneous tissue chambers *in vivo*, where an extensive, dense capsule was obtained after two hours incubation and a sparse capsule with thin fibers, after six hours incubation (Brogden and Clarke, 1997). Morck *et al* (1988) showed that the capsule was 4 - 5x thicker when recovered from an infected calf by pulmonary lavage than when grown *in vitro*, and it has also been demonstrated attached to alveolar epithelium (Morck *et al*, 1989) and to ovine lung surfactant *in vitro*, indicating that it may facilitate bacterial attachment to the lining of lung alveoli (Brogden *et al*, 1989).

The role of capsule in virulence of most of the *Pasteurellaceae* family has not been well studied, and in those few organisms where some study has been done, it has been found that encapsulation alone may not be sufficient to make the bacterium virulent. For instance, there are six encapsulated serovars of *H. influenzae,* yet type b accounts for more than 95% of systemic infections in children and is the most resistant to complement killing. However, mutation in genes responsible for type b expression result in loss of virulence (Kroll and Moxon, 1988), and when noncapsulated mutants express type b capsule following cloned DNA transformation, they are more virulent than mutants expressing other capsule types (Moxon and Vaughn, 1981). Thus, the structure and /or composition of the capsule may be important. The amount of capsule produced and its adherence to the bacterium may also influence the virulence of a particular strain. The total amount of cell-associated capsule in *H. influenzae* type b is associated with greater serum resistance (Sutton *et al*, 1982), although isogenic mutants producing half as much capsule are still virulent in animal models (Kroll and Moxon, 1988).

It has been found that a high serum antibody response to purified *P. haemolytica* A1 capsular polysaccharide (Adlam, 1989) correlates with resistance to transthoracic challenge with virulent *P. haemolytica* A1 in cattle (Confer *et al*, 1989), but whether *P. haemolytica* capsular material plays any role in URT adherence or not, is unknown. A few studies do indicate that *P. haemolytica* may adhere to the carbohydrate chains of mucins. Uhlich *et al* (1993) found that *P. haemolytica* A1 adhered to bovine nasal mucus *in vitro*, and that enzymatic degradation of mucus carbohydrates and proteins reduced adherence. Another research group found that capsular extracts of *P. haemolytica* A1 bound to preparations of bovine tracheal mucus (Botcher *et al*, 1993). This type of

adherence may play a role in early, low number colonization of the upper respiratory tract, and actually may enhance clearance of the bacteria.

c) Lipopolysaccharide

Lipopolysaccharide (LPS) or endotoxin produced by gram-negative bacteria are composed of the biologically active lipid A, core polysaccharide, and an antigenic polysaccharide chain (O antigen). Conserved as well as unique epitopes are present in the LPS of the *Pasteurellaceae* family, but these vary in stability depending on stage of growth, environmental conditions, and host-induced modification. A number of studies on the structure of LPS of *P. haemolytica* have been completed, and there appears to be a degree of variability in the LPS between serovars of *P. haemolytica*. A study in which water-extracted *P. haemolytica* strains were compared by crossed immunoelectrophoresis demonstrated serological differences among LPS of serovars 1 - 15 (Tsai *et al*, 1988). Although O antigens have generally been characterized as repeating oligosaccharide units, serovars vary in the sugar compositions of these chains. The chemical structure of the O antigen of *P. haemolytica* A1 LPS has been determined to be a trisaccharide repeat of two D-galactose residues and one N-acetyl-D-galactosamine residue (Severn and Richards, 1993). In general, serovar A strains appear to be rough, whilst T serovars appear to be smooth (Knights, unpublished). The fact that galactose is prevalent in *P. haemolytica* surface polysaccharides such as LPS and capsule lead to the cloning and characterization of the galE locus of serovar A1 (Potter and Lo, 1996). Gal E is the enzyme (UDP-galactose 4-epimerase) that is involved in galactose metabolism. Mutants defective in galE have been shown to exhibit reduced virulence, and thus are potential

vaccine candidates. Whether a *P. haemolytica* galE mutant would produce less LPS and capsule resulting in decreased URT adherence, is unknown.

Biologically, *P. haemolytica* LPS appears to have the same endotoxic activities as other gram negative LPS. Purified LPS from serovar A7 was shown to be as potent as LPS from *Salmonella typhimurium* and *Serratia marcescens* when subjected to different toxicity tests (Rimsay *et al*, 1981). This verified earlier data obtained by Keiss *et al* (1964) in which *P. haemolytica* LPS injected into sheep produced deleterious *in vivo* effects. Many more recent studies on serovar A1 LPS have been conducted in calves (Emau *et al*, 1986 and 1987) and sheep (Brogden *et al*, 1984 and 1986). The dose-dependent effect on peripheral bovine blood leukocyte function has also been studied (Confer and Simons, 1986). More recently, it has been found that LPS plays a role in leukotoxin function, and this may be important in bacterial virulence (Li and Clinkenbeard, 1999). Although many *P. haemolytica* vaccines contain LPS, vaccination and challenge experiments in calves have revealed no correlation between serum antibody levels to LPS and protection (Confer *et al*, 1986 and 1987).

Thus, LPS probably plays a role in the high mortality observed in acute pasteurellosis, and it appears that the majority of its effects are in the lung. Whether it plays any role in colonization of the URT, specifically in adherence to either mucus or the epithelial cell surface, is unknown.

## d) Outer Membrane Proteins

Very little is known about the function of outer membrane proteins of *P. haemolytica*. In a related bacterium, *Haemophilus influenzae*, that usually colonizes the

human respiratory tract via pili, high molecular-weight proteins have also been identified as adhesins by using a pili-deficient mutant in adherence studies (St. Geme and Falkow, 1990; Read *et al*, 1991). It is certainly possible that *P. haemolytica* may bind to epithelial cells via nonpilus adhesins yet to be elucidated. Protein profiles have been obtained for *P. haemolytica* envelope preparations and extracts of killed cells. A similar profile was seen for all strains of the A serovar, and these differed from the profiles of T serovar strains (Knights *et al*, 1990; Thompson and Mould, 1975). Donachie *et al* (1984) confirmed the OMP profiles of A serovars, and also found that A6 strains induced cross-protection in mice against A1 challenge. Saline extractable proteins from serovar A1 have been purified by Lessley *et al* (1985) and McKinney *et al* (1985) and they identified 15 proteins, of which 2 appeared to be protective in mice. However, in further experiments in which cattle were immunized, Confer *et al* (1985) suggested that capsular polysaccharide in the fractions may have been responsible for the protective properties.

Extracts of intact *P. haemolytica* A1 used for preparations designed to provide immunoprotection against infection had been used for many years before a detailed analysis characterizing these extracts was performed. Squire *et al* (1984) separated the inner and outer membrane protein from an A1 serovar strain, producing two major OMPs with molecular masses of 30kDa and 42 kDa. Five Sarkosyl-soluble proteins were produced from the inner membrane. That these proteins might have biological relevance led Craven *et al* (1991) to clone a segment of DNA from *P. haemolytica* A1 using recombinant DNA techniques, that encoded three proteins of 28, 30, and 32 kDa. The 28 and 30 kDa proteins reacted on a Western blot with bovine serum raised against live *P. haemolytica* A1 cells, and cattle vaccinated with the cloned 30 kDa protein developed

antibodies which correlated with resistance to challenge. Morton *et al* (1995) also demonstrated homologous protection using Sarkosyl extracts of *P. haemolytica* A1, A6, and A9.

From these studies, it was thought that the 30 kDa protein was probably an immunity-inducing surface antigen of *P. haemolytica* A1. As this protein had potential as a possible virulence factor, further work was done by Murphy and Whitworth (1993) and Cooney and Lo (1993). Sequence analysis revealed that 3 separate genes encoding 3 lipoproteins (lpp1, lpp2, and lpp3), were tandemly arranged on *P. haemolytica* A1 chromosome and transcribed from a single promoter. These proteins were shown to have homology with a 28 kDa inner membrane lipoprotein of *E. coli* and a 28 kDa membrane lipoprotein of *H. influenzae* type b, that appeared to contribute to virulence in a rat challenge model. The 3 genes were then separately cloned into an expression vector, and recombinant forms of the 3 proteins were purified after expression in *E. coli*. Using Western blot, a correlation between antibodies to lpp3 and resistance to challenge of cattle by *P. haemolytica* was found (Dabo *et al*, 1994). From 1990 to 1994, molecular genetic techniques for *P. haemolytica* were being developed and refined, and in 1994, an isogenic mutant of *P. haemolytica* A1 that no longer synthesized these 3 proteins was constructed using allelic replacement (Murphy and Whitworth, 1994). This mutant was less virulent than the wild-type for mice and was more susceptible to complement-mediated killing. Whether this surface protein plays any role in bacterial adherence is unknown. This also holds true for the few other OMPs that have been identified as immunogens thus far. These include a 60 kDa protein (Lo and Mellors, 1996), a 38 kDa protein (Pandher and Murphy, 1996), a 35 kDa (PomA) and 32 kDa (Pom B) protein

(Mahasreshti *et al*, 1997), and serotype-specific antigen (Gonzalez *et al*, 1995). Also, Frank *et al* (1994) reported a correlation between antibody against a 94 kDa membrane glycoprotein and resistance to nasal challenge. Morton *et al* (1994) also identified a 94 kDa protein that did not correlate with resistance to intrathoracic challenge, which may indicate that this protein is active in URT colonization, and not LRT infections.

e)  Secretory Proteins: Leukotoxin (LKT), neuraminidase, and sialoglycoprotease.

Immunization of cattle with culture supernatant proteins, harvested from log phase cultures of *P. haemolytica* A1, has been shown to induce protection against experimental challenge with live *P. haemolytica* A1 (Shewen and Wilkie, 1988). This organism secretes a number of proteins into culture medium, including LKT, neuraminidase, proteases, and a number of soluble surface antigens, including serotype-specific antigens, that are probably shed during bacterial growth. Some or all of these obviously play a role in the pathogenicity of BRD, but whether any are significant in the initial adherence and colonization of *P. haemolytica* to the URT of cattle is unknown.

All known serovars and several untypable strains of *P. haemolytica* isolated from cattle produce LKT during the logarithmic growth phase (Shewen and Wilkie, 1985). In culture supernatants, native LKT is found as a multiprotein complex (>700 kDa), with the toxic unit being a protein of 105 kDa that is degraded to proteins of 97 and 66 kDa (Chang *et al*, 1987). *Pasteurella haemolytica* LKT is a pore-forming cytolysin that damages the plasma membrane of bovine neutrophils (Clinkenbeard *et al*, 1989) and alveolar macrophages (Markham and Wilkie, 1980), and has more recently been found to cause cytolysis of platelets (Clinkenbeard and Upton, 1991). The inflammatory response that ensues causes tremendous damage to the bovine lung. The details of the lung

response to LKT are beyond the scope of this review, and very little is known about the role of this virulence factor in the URT. Data obtained from challenge experiments in cattle suggest that LKT is produced *in vivo* and that it evokes the production of neutralizing antibodies (Moore *et al*, 1985). Thus, LKT is probably produced as the bacteria replicate to high numbers on the nasopharyngeal surface, but whether this causes epithelial cell damage aiding in adherence and/or progression of bacteria down the respiratory tract, or merely functions as part of the bacterial protective mechanism against mucosal immunity is unknown.

Bacterial neuraminidases are heat-labile, extracellular enzymes produced maximally in the stationary phase of *P. haemolytica* growth that remove sialic acid from mucus or cell surface glycoproteins. In doing so, they reduce the protective effects of mucus by decreasing viscosity and possibly enhancing the adherence of *P. haemolytica* to the epithelium by exposing host cell receptors. The production of neuraminidase by three of five untyped strains of *P. haemolytica* was first demonstrated in 1970 (Scharmann *et al*) This was later confirmed to be produced by all 16 serovars, except *P. haemolytica* A11 and T serovar strains (Frank and Tabatabai, 1981; Straus *et al*, 1993). Little neuraminidase has been found in the culture supernatant, with most appearing to be cell-associated (Frank and Tabatabai, 1981). However, Otulakowski *et al* (1983) found this enzyme in a crude preparation of culture supernatant cytotoxin. It was thought that a possible relationship may exist between neuraminidase activity and cattle pneumonia, as serovar A1 had a higher neuraminidase activity than serovar A2 (Frank and Tabatabai, 1981), but a later study by Straus and Purdy (1995) on a far larger number of field isolates, showed that serovar A2 produced significantly more neuraminidase than serovar

A1. All strains examined were from sick animals, so it could still be possible that the enzyme is required to cause pneumonic infections, regardless of serovar.

A neutral glycoprotease specific for O-sialoglycoprotein on human erythrocytes was demonstrated (Otulakowski *et al*, 1983; Abdullah *et al*, 1992), and identified for 9 of 12 *P. haemolytica* serovars, including A1 (Abdullah *et al*, 1990). The gene from serovar A1 has been cloned, sequenced, and expressed (Abdullah *et al*, 1991), and antibodies to the A1 sialoglycoprotease have been identified in calves after intrapulmonary challenge (Lee *et al*, 1994). The role of this enzyme in URT colonization is unknown, but it may play a similar role to that of other proteases which degrade fibronectin, possibly exposing potential binding sites.

## Host Factors involved in Adherence

### a) Fibronectin

Bacterial adherence need not be directly to the host cell surface. Two well known gram positive pathogenic bacteria, *Streptococcus pyogenes* and *Staphylococcus aureus* adhere to fibronectin (Courtney *et al*, 1986; Froman *et al*, 1987), which is a large (molecular weight of 440,000) extacellular matrix and plasma glycoprotein, that has adhesive functions such as cell-to-cell attachment, cell adherence to basement membranes, and clot stabilization. Binding of the amino-terminal domain of fibronectin to various *Salmonella* strains has also been reported (Baloda *et al*, 1985). However, in recent studies, it has been shown that fibronectin on the surface of oral epithelial cells blocks the binding of gram-negative bacilli such as *Pseudomonas aeruginosa* and *Escherichia coli* (Simpson *et al*, 1985; Abraham *et al*, 1983), and blocks the binding of *P. aeruginosa* to tracheal epithelium (Ramphal and Pyle, 1985). Thus, in colonization of the

upper respiratory tract, there appears to be an inverse relationship between cell-surface fibronectin and adherence of gram-negative bacilli. It is also known that stress (Woods, 1987) and cytolytic and non-cytolytic viral infections (Ramphal *et al*, 1980; Proctor, 1987), result in decreased levels of fibronectin, promoting growth of gram-negative over gram-positive bacterial populations (Johanson *et al*, 1980; Woods, 1987).

The decrease in fibronectin, which coincides with increased bacterial adherence, is thought to be due to proteolytic activity. This activity can either result in unmasking of host cell surface molecules in the upper respiratory tract allowing specific antigens to adhere at higher efficiency and providing a competitive advantage (Whiteley *et al*, 1992), or unmasking of host cell surface molecules such as integrin or laminin, which bind with lower affinity to a more ubiquitous adhesin of *P. haemolytica*. The latter could be the stimulus for synthesis or expression of another adhesin for higher affinity binding (Finlay and Falkow, 1989; Valkonen *et al*, 1991; Hoepelman and Tuomanen, 1992). The proteolytic activity is probably due to host or bacterial proteases which are able to degrade fibronectin (Yamada, 1983; Woods *et al*, 1980). One such protease found in increased quantities in saliva of human patients recovering from gram-negative bacillary pneumonia is leukocyte elastase (Dal Nogare *et al*, 1987). As cattle show a marked influx of leukocytes to the URT in IBR infection, Briggs and Frank (1992) conducted an experiment demonstrating an increased elastase activity in nasal mucus, which occurred after calves were exposed to IBR virus. The increase in elastase preceded *P. haemolytica* colonization and elastase levels decreased as colonization by *P. haemolytica* decreased.

b) Mucus

Mucin is a high molecular weight glycoprotein composed of a central rod-like peptide core that produces the high viscosity and elasticity of mucin, and branching oligosaccharides that can potentially bind bacteria and toxins. Mucin usually forms cross-linked aggregates to give a gelatinous layer on a mucosal surface. This layer is

composed of two parts: a superficial layer of true mucin secreted by Goblet cells, and a deeper, serous layer secreted by submucosal serous cells. The latter bathes the cilia and contains products that play a role in local immunity, such as lactoferrin, lysozyme, and secretory IgA.

The mucociliary cleansing mechanism is an important early defense mechanism of the URT. Several mechanisms have been proposed to explain the ability of mucus to protect the underlying epithelium. The simplest mechanism is that mucus provides a physical barrier which traps bacteria and prevents their attachment to underlying cells. Another possibility is that polysaccharides of mucus-type glycoproteins contain identical carbohydrate sequences found on membrane receptors for bacteria. These soluble receptors bind to bacteria and prevent their attachment to surface cells. A third potential protective mechanism involves increased secretion of mucin which engulfs pathogens and transports them out of the airways (Lamont, 1992 ). However, if the mucus is altered during a disease process or during stress, it appears that, in most cases, bacteria can more easily contact the underlying epithelium to adhere and colonize.

Many different microorganisms demonstrate an affinity for mucus, and there is preliminary evidence shown by ELISA, that *P. haemolytica* adheres to bovine nasal mucus (Uhlich *et al*, 1993), and that adherence can be altered by enzymatic degradation of both carbohydrate and protein components of mucus. These experiments were performed *in vitro*, and bacteria appear to adhere less to mucin when it is stationary in a microtiter plate than in solution, such as would occur *in vivo*. Thus, structural conformation of the mucin appears to play an important role in adherence. It has also been shown that OMP preparations, capsular extracts, and whole cell suspensions of *P. haemolytica* bind to preparations of bovine tracheal mucus (Botcher *et al*, 1993). Thus, it appears that *P. haemolytica* adheres to mucus lining the URT of cattle, although this may occur only in the healthy host when *P. haemolytica* numbers are low.

The most studied of closely related organisms is *Haemophilus influenzae*. This organism appears first to bind avidly to mucus before adhering to damaged ciliated or unciliated epithelial cells (Read *et al*, 1991; St. Geme and Falkow, 1990; Stephens and Farley, 1991; Davies *et al*, 1995). Potential receptors for adherence of this organism to mucus are questionable, although Reddy *et al* (1996) have found that binding appears to be mediated by outer membrane proteins P2 and P5 of the bacteria and sialic acid-containing oligosaccharides of mucin. The only similar organism for which receptors have been partially elucidated is *Pseudomonas aeruginosa*, which binds to dissacharide units of the oligosaccharide side-chains of mucin. Sialic acid also seems to play a role in that it appears to maintain the conformation of the mucin, but it does not react directly with the bacterial adhesin.

A *P. haemolytica* adhesin to mucus has yet to be found if indeed adherence to mucus does occur *in vivo*; it appears that capsule is the most likely candidate, but fimbriae and/or outer membrane proteins may also play a role. It is thought that binding is one of a nonspecific, hydrophobic interaction.

## Methods and Models of Bacterial Adherence

Over the last 25 years, concern with pathogenesis of bacterial infections has led to a growing interest in adherence of bacteria to tissue surfaces. This, in turn, has evoked the development of a number of models to study adherence of bacteria to various nonphagocytic cells and to look more closely at bacteria-host cell interactions in a number of diseases. Three types of mammalian cells have been used in these models: Cells from intact excised tissue (organ cultures or explants) (Read *et al*, 1991; Dugal *et al*, 1992), tissue culture cells (St. Geme and Falkow, 1990), and cells in suspension

obtained from scraped tissues or exfoliated cells (St. Geme and Cutter, 1996; Schaeffer *et al*, 1979). In all these models, adherence assays are performed by incubating bacterial suspensions with mammalian cells and then separating cells with adherent bacteria from nonadherent bacteria. Many techniques have been used for separation, including sequential washing (St. Geme, 1994), filtration (Bakaletz *et al*, 1988), and differential (Beachey and Ofek, 1976) or density gradient centrifugation (Valentin-Weigand *et al*, 1987). Likewise, many techniques have been employed for quantitation of adherent bacteria or determination of bacterial adherence, including direct microscopic counting (Bakaletz *et al*, 1988), enumeration of CFU (St. Geme and Cutter, 1996), counting radiolabeled or fluorescent-dye labeled bacteria (St. Geme, 1994), and reading absorbance of a color change in enzyme-linked immunosorbent assays (ELISAs) (Ofek *et al*, 1986).

Thus, in choosing an appropriate method to determine bacterial adherence, each researcher has to make decisions based on the availability of mammalian cells, the reliability of the technique used to separate adherent from nonadherent bacteria, and the sensitivity and precision of the quantitation technique. The fact that different assay results have exhibited considerable variability both between and within laboratories, does lend credence to the fact that each method is unique in its advantages and limitations (Mackowiak and Marling-Cason, 1984).


Comparison of Mammalian Cell Types used in Adherence Assays

Obviously the optimal system in which to study adherence is *in vivo*, where experimental animals are inoculated, sacrificed, and then tissue removed to determine

adherence. This type of system has all the advantages of mimicking the natural disease situation, but it does suffer from many disadvantages, not least of which are that the disease must be easily and reliably induced in the animal, the animal population must be large enough to provide statistical validation of the data, and these animals must be sacrificed. Unfortunately, all of these criteria are severely limiting when working with cattle. The sole use for this system to date has been in laboratory animals and pigs to confirm data obtained from other models using artificial targets (Aronson *et al*, 1979; Dom *et al*, 1994). In cattle, *in vivo* models have only been developed for the experimental production of pneumonic pasteurellosis in order to study pathogenesis and to test the efficacy of immunization and treatment. These models have involved inducing the disease either by stressing the calf and infecting with viruses (Jericho *et al*, 1976), or by damaging the protective mechanisms of the URT (Gibbs *et al*, 1984), or by intrathoracic inoculation, bypassing the URT (Panciera and Corstvet, 1984). In all of these models adherence to the URT mucosa has not or cannot be studied.

Intact excised tissue in the form of organ cultures or explants more closely mimics epithelial cells as they are found *in vivo* than any of the other cell types, and therefore, are well suited to adherence studies. This model however suffers from limited sources such as biopsies. Therefore, most experiments have involved the use of intestinal tract mucosal segments (Baselsky and Parker, 1978; Zilberberg *et al*, 1983), although, more recently, experiments have been conducted using respiratory tract mucosa, including tracheal rings (Bélanger *et al*, 1990; Jacques *et al*, 1993; Ramphal and Pyle, 1983) and upper respiratory tract epithelium (Read *et al*, 1991, Feldman *et al*, 1992; Dom *et al*, 1994). Nasal, nasopharyngeal, turbinate, or tracheal tissue have been taken from

freshly slaughtered animals and kept viable in tissue culture medium for the duration of an experiment. Provided tissue is undamaged and can be easily maintained for a short time, using actual bovine epithelial tissue has an advantage over the cell culture method where a monolayer of a cell line is used. In the tissue culture model, cell types available are not always appropriate in that they are usually undifferentiated, nonpolarised, often have surfaces distinct from their origin especially in terms of distribution and accessibility of receptors, and are often obtained from a different tissue to the one being studied. The tissue culture method also requires adequate tissue culture facilities and maintenance of culture preparations, but once this is set up, regular splitting of cells becomes routine, and it is easier to obtain cells for an experiment than from a new slaughtered animal each time an experiment is to be conducted. The tissue culture system may be very useful when a specific issue needs to be addressed in defining the type of receptors for bacterial adhesins. For example, the role of carbohydrates in adherence was studied by treating tissue culture cells with an inhibitor of protein glycosylation (Elbein *et al*, 1981), and the role of fibronectin in *Streptococcus pyogenes* adherence was determined by employing tissue culture cells with varying amounts of fibronectin on their surfaces (Stanislawski *et al*, 1985).

Perhaps the most useful model when the study involves the property of adherence to tissues normally colonized by the test bacteria, is scraped tissue epithelial cells or exfoliated cells, in suspension. Scraping cells from tissue can provide a large number of cells from a variety of tissues. Ease of collection has made this a widely used method that began with the pioneering work of Ellen and Gibbons (1973) who studied the adhesion of *S. pyogenes* to scraped buccal epithelial cells. However, it is difficult to

maintain the viability of these cells, and they usually harbor normal flora which may occupy sites for the test bacteria. Another disadvantage of this model is that adherence of mucoid strains of bacteria such as *Pseudomonas aeruginosa* (Ramphal and Pyle, 1983) or *Haemophilus influenzae* type b (Read *et al*, 1991) does not readily occur, perhaps because of the lack of overlying mucin, thought to play a role in adherence, which is found *in vivo* and can often be found in strands on uninjured organ cultures. Another disadvantage of the scraped epithelial cell method is that nonluminal surfaces are available for attachment of bacteria, unlike in the excised tissue or cell line monolayer, where the underside of the tissue is unavailable for adherence.

## Comparison of Methods for Separation of Adherent from Nonadherent Bacteria

Most procedures used to separate adherent from nonadherent bacteria are rapid and effective. Sequential washing is the most widely used method to remove nonadherent bacteria from an organ culture, cell monolayer, or immobilized epithelial cells, such as in ELISAs. However, in this method some weakly bound bacteria may be removed by shear forces, and it is usually impossible to establish the extent of this loss which may be considerable (Ofek and Doyle, 1994). Shear forces are also a problem when using differential centrifugation or filtration and washing of scraped epithelial cells. The use of density gradients to separate cell-adherent from nonadherent bacteria avoids the shear problem (Valentin-Weigand *et al*, 1987). However, this method is restricted to epithelial cell suspensions, and it is unknown whether the gradient medium has an effect on adherence. Posing the opposite problem to the loss of adherent bacteria, fixed surfaces have imperfections and nonadherent bacteria easily become trapped in fissures

or damaged areas and are difficult to remove. This may be one case where shear could be advisible.

When target cells are immobilized to a surface using polylysine or lysine and glutaraldehyde, or when a monolayer or organ culture is used, there has to be a means to reduce nonspecific, random adherence to the surfaces not occupied by the host cells. Blocking agents that do not react with bacteria and have a high affinity for tissue cells are used; for example, bovine serum albumin, gelatin, hemoglobin, or Tween 20. Gentle washings of these surfaces usually remove 80 - 99% bacteria (Ofek and Doyle, 1994).

Filtration and differential centrifugation methods suffer not only from problems of shear, but also from high backgrounds of nonadherent bacteria. This may be largely due to clumping or chain formation of exponential phase bacteria, and may be overcome with prefiltering or precentrifugation.

Comparison of Methods for Quantitation of Bacterial Adherence

Determination of bacterial adherence depends on the model system used, some of which yield a qualitative estimate while others yield a quantitative value. Electron microscopic observations are valuable in determining the extent of adherence and site of adherence especially on excised tissue either as an organ culture or after *in vivo* inoculation. However, electron microscopy is not amenable to quantitation of bacterial-target cell interactions (Costerton, 1980). Bacterial labeling procedures using radioactive isotopes or fluorescent dyes can also be used in a qualitative assessment of percentage adherence. This can be achieved by determining radioactivity or fluorescence of adherent bacteria expressed as a percentage of the total radioactivity or fluorescence of

the inoculum incubated on the tissue before washing to remove nonadherent bacteria. The use of labeling either as a qualitative or quantitative determination has the advantage of being a very sensitive assay system that is widely used. There is a linear relationship between numbers of bacteria and radioactive/fluorescent counts which makes quantitation easily achieved. However, label is sometimes lost from bacteria due to metabolic turnover or lysis, and the use of fluorescent dyes which attach to bacterial surface proteins may physically block adhesins necessary for target cell interaction. Radiolabel also has the advantage over fluorescent dyes in that it is not prone to quenching with time, and can still be counted if hidden in tissue folds or cilia. The ELISA technique using immobilized scraped epithelial cells (McEachran and Irwin, 1986; Ofek *et al*, 1986) and tissue culture cells (Stanislawski *et al*, 1985) has also been introduced as a sensitive method to enumerate adherent bacteria. One advantage of this method is that all labeling occurs after adherence, so bacteria are not modified in any way during the adherence process. This method is also very sensitive because bound enzyme can produce substrate product continuously over a long period, is reproducible, and allows for a number of replicates in each experiment. However, several steps have to be standardized, and a standard curve needs to be generated with each experiment in order to equate bacterial numbers with color development.

Most tissue culture models utilize viable or microscopic counts for quantitation of bacteria. One disadvantage with viable counts (or determination of colony forming units/ml), is that host cells must be lysed to release adherent bacteria. This is usually done with Triton-X for 10 minutes, and there is some question as to whether cells are lysed sufficiently to separately release all bacteria. Also, a mixture of viable and dead

cells usually coexist once bacteria reach stationary phase, which can be when certain adhesins are expressed (Duguid and Old, 1980), and dead cells may adhere and would not be counted by the assay. Microscopic estimates of adherence, which are often used to enumerate bacteria adherent to tissue culture monolayers or scraped epithelial cells, reveal the presence of both dead and live bacteria, and the distribution of bacteria on target cells can be measured (Scaletsky *et al*, 1984). Microscopic counts to some extent enable the distinction between truly adherent bacteria and background nonadherent bacteria. This type of counting is arithmetic. Small differences in numbers of adherent bacteria are easily detected, but this method is also very tedious, time-consuming, and can be subjective.

# CHAPTER III

# HYPOTHESIS AND EXPERIMENTAL OBJECTIVES

The hypothesis upon which this research is based is that adherence of *P. haemolytica* serovar A1 to bovine URT tissues allows this bacterium to colonize and become established as the predominant serovar in BRD, thus promoting infection of the lung and development of fibrinopurulent bronchopneumonia.

Therefore, the <u>principal objectives</u> of this research were:

(1)     to develop and evaluate an *in vitro* fluorometric tissue culture assay appropriate for the investigation of adherence of *P. haemolytica* to bovine upper respiratory and epithelial cell lines.

(2)     to use the *in vitro* fluorometric assay to compare adherence of *P. haemolytica* serovar A1 to *P. haemolytica* serovar A2, and screen bacterial factors that are likely to influence adherence.

(3)     to develop an *ex vivo* model suitable for study of adherence of *P. haemolytica* to a variety of relevant mucosal tissues of the bovine URT, i.e. nasal, nasopharyngeal, turbinate, and tonsillar tissues, and to confirm that adherence occurs.

(4)    to use the *ex vivo* adherence model to investigate the regional adherence of *P. haemolytica* in the bovine URT and to test whether adherence is affected by capsule, serovar, and bovine herpes virus-1 (IBRV).

It is expected that the experimental techniques and knowledge derived from this research will facilitate further study of potential *P. haemolytica* adhesins and/or bovine URT epithelial cell receptors, and lead to a better understanding of the early pathogenesis of BRD. The ultimate goal is inhibition of early URT colonization by *P. haemolytica* A1 via appropriate management, treatment and/or protective vaccines.

CHAPTER IV

DEVELOPMENT OF AN *IN VITRO* FLUOROMETRIC ASSAY TO STUDY

ADHERENCE OF *PASTEURELLA HAEMOLYTICA* TO BOVINE CELLS

Introduction

*Pasteurella haemolytica* is the bacterium most frequently isolated from lungs of cattle suffering from acute bovine respiratory disease (BRD) (Frank, 1986 and 1989). *Pasteurella haemolytica* is considered a commensal organism in the upper respiratory tract of cattle. A low number of predominantly *P. haemolytica* serovar A2 (PhA2) can be detected in the nasopharynx of healthy, nonstressed cattle. Results of *in vivo* experiments indicate that pulmonary infection in cattle with BRD is preceded by rapid growth of *P. haemolytica* serovar A1 (PhA1) on the respiratory epithelium of the nasopharynx, followed by colonization of lungs via aerosolized droplets. These results suggest that PhA1 is better able to take advantage of stress-associated changes in the respiratory tract as a result of transport, inclement weather, or viral infection, than is the more ubiquitous PhA2 (Frank and Smith, 1983; Frank *et al*, 1986; Frank *et al*, 1987; Frank 1988).

Various methods have been used to study bacterial colonization of mammalian cells. *In vivo* field studies that involve collecting specimens of nasal secretions (Frank *et al*, 1986, 1987, 1989, and 1994) and tonsillar washes (Frank *et al*, 1994; Frank and Briggs, 1992) from calves before, during, and after shipping, followed by isolation of

bacteria by use of bacteriologic culture techniques, have provided important information on the role that *Pasteurella* spp. play in BRD. These results have led to the conclusion that PhA1 has virulence factors that allow it to become the predominant serovar in acute BRD (Frank, 1988). The ability of PhA1 to preferentially multiply on respiratory epithelium of the nasopharynx of stressed cattle and to evade the mucociliary clearance mechanism may be attributable to adherence of this serovar to respiratory mucosa.

The complexity of the upper respiratory tract mucosal microenvironment complicates *in vivo* evaluation of bacterial adherence. Thus, study of specific interactions between PhA1 and mammalian host cells may best be accomplished by use of *in vitro* techniques. Such techniques allow for isolation of various factors that may facilitate colonization by PhA1, and the study of these factors at the molecular and cellular level. Generally, *in vitro* adherence assays involve incubation of bacteria with host cells, followed by removal of nonadherent bacteria, lysis of host cells with detergent to release adhered bacteria, and culture of lysate dilutions to enumerate bacteria. These assays can be time consuming and labor intensive. The use of a fluorescent probe to directly measure the attachment of labeled bacteria to intact host cells would avoid lysis of host cells and subsequent culture of bacteria, and may thus provide an accurate and labor-efficient approach to the study of bacterial adherence. Therefore, the objectives of the study reported here were to develop an *in vitro* fluorometric assay to assess *P. haemolytica* adherence to bovine respiratory and epithelial cells, to compare adherence of PhA1 and PhA2, and to test whether *P. haemolytica* capsule and outer membrane protein (OMP) preparations had an effect on adherence.

Materials and Methods

Bovine Cell Lines

Two commercially available cell lines were used in this study. Madin Darby bovine kidney (MDBK) cells (ATCC #CCL-22; American Type Culture Collection, Manassas, VA) were used for development of the *in vitro* fluorometric adherence assay, and MDBK and bovine turbinate (BT) cells (ATCC #CRL-1390; American Type Culture Collection, Manassas, VA) were used to compare PhA1 and PhA2 adherence, and to test the effect of capsule and outer membrane proteins on adherence of PhA1 and PhA2. Cells were maintained in minimum essential medium with Earle's balanced salts, 2.0 mM L-glutamine, 8% fetal bovine serum, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 100 U/ml) (Sigma Chemical Co.). Based on results of preliminary experiments that indicated that nonspecific binding of bacteria to tissue culture plate surfaces was reduced by > 80% when wells were coated with gelatin and that such coating did not affect establishment of MDBK cell monolayers, 200 μl of 0.1% porcine skin gelatin (Sigma Chemical Co.) was added to each well of 96-well, flat-bottom tissue culture plates (Cell Wells™, Corning Glass Works), and plates were incubated at 37 C for 1 hour. After draining the gelatin solution and air-drying the plates, trypsinized suspensions of either MDBK or BT cells were added to the wells and incubated in antibiotic-free tissue culture medium (ie, minimum essential medium with Earle's balanced salts, 2.0 mM L-glutamine, and 8% fetal bovine serum) at 37 C in 5% $CO_2$ until confluent monolayers were formed, usually at 72 hours. Monolayers of cells were washed twice with 0.3 ml of phosphate buffered saline solution (PBS; pH 7.2 to 7.4)

per well, and the final wash buffer was aspirated from wells immediately before monolayers were used in the adherence assay.

## Development of the *in vitro* Fluorometric Adherence Assay

*Pasteurella haemolytica* serovar A1 strain 668 (supplied by Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK), a low passage strain isolated from the lungs of a calf with BRD, was used to develop the assay. Bacteria were labeled by use of a procedure described by Geelen *et al* (1993). Briefly, PhA1 was cultured on blood agar for 18 hours at 37 C, harvested in PBS, and diluted with an equal volume of 2 mg/ml of fluorescein-5-isothiocyanate (FITC)/ml (Sigma Chemical Co., St. Louis, MO) in 0.05 M sodium carbonate and 0.1 M sodium chloride buffer (pH, 9.11). Bacteria and FITC were incubated for 1 hour at 4 C, and bacteria were then washed with 10 ml of PBS by centrifuging at 11725 g for 10 minutes. The final pellet was resuspended in 2 ml of PBS and then diluted in PBS to the desired bacterial concentration. To determine the optimal number of washes required for removal of unbound FITC, fluorescence of supernatants (recorded as fluorescent units, which are scaled instrument response values) collected after each centrifugation of a range of concentrations of PhA1 ($10^6$, $10^7$, and $10^8$ colony-forming units [CFU]/ml) was determined with a spectrofluorometer (Cytofluor 2300 Fluorescence Measurement System, Millipore Corp.).

Fifty microliters of FITC-labeled PhA1 in antibiotic-free tissue culture medium was added to each well of washed monolayers of MDBK cells. Plates were incubated at 37 C in 5% $CO_2$ for various times. After incubation, monolayers were washed 5 times

with PBS to remove nonadherent bacteria, wells were refilled with 200 μl of PBS, and fluorescence was measured with a spectrofluorometer.

To identify the optimal concentration of FITC-labeled PhA1 that would provide enough fluorescence to allow detection of a low degree of adherence, 50 μl of 4 concentrations of labeled PhA1 ($5 \times 10^4$, $5 \times 10^5$, $1 \times 10^7$, and $8 \times 10^8$ CFU/ml) was initially incubated with MDBK cells, and fluorescence of adhered bacteria was measured. Fluorescence was correlated with number of adhered bacteria (CFU/ml) determined by use of a quantitative bacteriologic culture technique. The quantitative technique that was used involved releasing MDBK cells with the addition of 300 μl of 0.5% Triton-X 100/well (Sigma Chemical Co.) for 5 minutes, followed by determination of CFU of PhA1/ml of lysate by use of the Miles-Misra technique (Quinn *et al*, 1994).

The optimal incubation time necessary for adherence of bacteria to cell monolayers was determined by incubating MDBK monolayers with 50 μl of FITC-labeled PhA1 ($1 \times 10^8$ CFU/ml) for 15, 30, 45, 60, 90, 120, 150, and 180 minutes (n = 6 wells/time). Number of bacteria that adhered to cell monolayers at each time was determined fluorometrically and by use of quantitative bacteriologic culture.

Fluorescein-5-isothiocyanate is a small molecule (molecular weight, 389) that binds to bacterial outer membrane amine groups (Haugland, 1996). To evaluate whether FITC interfered with the interaction between bacteria and bovine cells by blocking potential binding sites, adherence of FITC-labeled and nonlabeled PhA1 organisms to MDBK cells (n = 5 wells/group) were compared. Adherence was estimated by quantitative bacteriologic culture.

## Comparison of Adherence of PhA1 and PhA2

To provide preliminary information on serovar-dependent adherence, fluorometric adherence assays were performed using MDBK and BT cell lines, and FITC-labeled PhA1 strain 668 and FITC-labeled PhA2 strain D7 (Supplied by Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK). *Pasteurella haemolytica* serovar A2 strain D7 was labeled with FITC as described for PhA1, strain 668. Each well of bovine cell monolayers was inoculated with 50 $\mu$l of 1 x $10^8$ CFU of FITC-labeled bacteria/ml (n = 5 wells/group) and incubated for 45 minutes at 37 C in 5% $CO_2$. Duplicate sets of wells were inoculated with labeled bacteria so that, after incubation, 1 set of wells was washed 5 times with PBS to remove nonadherent bacteria and 1 set was not washed. Fluorescence in washed wells and unwashed wells was determined, and the percentage of bacteria adhered (percentage of adherence) was calculated as follows:

% adherence = (Fluorescence in washed wells/fluorescence in unwashed wells) X 100

Negative control samples included wells of MDBK and BT cell monolayers incubated with 50 $\mu$l of supernatant from the final wash after FITC-labeling of bacteria, FITC-labeled bacteria added to wells without MDBK or BT cells, and wells that contained only PBS. The experiment was repeated three times.

Percentage of adherence of PhA1 and PhA2 to MDBK and BT cells was compared by use of a 2 x 2 factorial arrangement of treatments with repeated experiments represented as blocks. Significance was set at $P < 0.05$.

## Effect of PhA1 Capsule on Adherence

Six hour Brain-Heart-Infusion (BHI)-broth cultures of PhA1 and PhA2 were shown to contain bacterial cells with a large amount of capsule as determined by a

Quellung reaction using hyperimmune rabbit sera to both PhA1 and PhA2. In contrast, bacteria grown for 18 hours demonstrated little or no capsule. Using similar initial inoculum concentrations of both 6- and 18-hour unlabeled cultures of PhA1 and PhA2, adherence to MDBK and BT monolayers was determined using direct quantitative culturing. To ascertain that the changes in adherence were due to the presence of capsule and not other differences in 6- and 18-hour cultures, capsular material was also stripped from 6-hour cultured cells using a saline capsule extraction (Gentry et al, 1982), and compared to 6-hour capsulated cultures.

Effect of Blocking with Sarkosyl-derived PhA1- and PhA2-OMPs on Adherence

Sarkosyl-derived OMP preparations of PhA1 strain 668 (22.44 mg protein/ml) and PhA2 strain D7 (8.92 mg protein/ml) were used at concentrations of 0, 25, 75, and 150 ug/ml to test their ability to interfere with binding of bacteria to MDBK and BT cells in a competitive assay. Bacteria were fluorescein-labeled as before, and bovine cells were incubated with various concentrations of OMP preparations for 1 hour at 37C before being washed 3x with PBS to remove excess OMP. The assay was performed as previously described, and bacterial adherence to washed bovine cells was compared to that of unwashed cells, and expressed as a percentage adherence of total bacteria added.

Results

*In vitro* Fluorometric Adherence Assay

During FITC-labeling of PhA1, we determined that 3 washes with 10 ml of PBS were adequate to remove most of the unbound FITC (Fig 1). Thus, for subsequent

assays, we consistently used 3 washes. The small amount of unbound FITC that remained in the supernatant after 3 washes did not appear to bind to MDBK cells, as indicated by the fact that fluorescence determined after incubation of this supernatant with MDBK cells for 45 minutes followed by washing cells 5 times to remove nonadherent FITC was not significantly greater than that determined for sterile PBS.

Number of PhA1 organisms that adhered to MDBK cells increased when inoculum concentration increased (Fig 2). Fluorescence of adhered bacteria was low and not easily detectable at inoculum concentrations $\leq 1 \times 10^7$ CFU/ml. Therefore, the optimal concentration of bacteria selected for use in subsequent fluorescence adherence assays was $1 \times 10^8$ CFU/ml, which is similar to the concentration of bacteria typically used by other researchers performing different types of *in vitro* adherence assays (Davies *et al*, 1995; St. Geme and Cutter, 1996).

Determination of effects of incubation time on adherence indicated that adherence of PhA1 to MDBK cells was clearly measurable by 45 minutes (Fig. 3). However, comparison of results obtained fluorometrically and by use of quantitative bacteriologic culture revealed different patterns of adherence. Except for a slight decrease at 60 minutes, the number of adherent organisms measured by use of quantitative culture increased progressively over time. This increase may have been attributable to bacterial replication, which would result in an increased number of unlabeled daughter cells. Fluorometric estimation of adherence indicated that adherence (ie, fluorescent units) reached a maximum at 1 hour and remained constant thereafter. Estimation of adherence by use of fluorometric techniques is less likely to be affected by bacterial replication than estimation by use of quantitative bacteriologic culture, provided that replication rate is

low and unlabeled daughter cells do not compete with labeled cells. To avoid any effects that increasing numbers of unlabeled organisms would have on estimation of adherence, the shortest incubation time that resulted in fluorometrically measurable adherence (i.e. 45 minutes) was selected as the optimal incubation time.

Adherence of nonlabeled and FITC-labeled PhA1 organisms to MDBK cells was not significantly different, which indicated that binding of FITC to bacterial outer-membrane amine groups did not affect interactions between bacteria and bovine cells.

Comparison of PhA1 and PhA2 Adherence

Factorial analysis revealed significant ($P < 0.001$) effects of bacterial serovar and bovine cell type on adherence. Mean ($\pm$ SD) percentages of adherence of PhA1 to MDBK and BT cell monolayers ($3.64 \pm 0.09\%$ and $4.97 \pm 0.48\%$, respectively) were greater than that of PhA2 ($2.93 \pm 0.17\%$ and $3.49 \pm 0.70\%$, respectively). In addition, percentage of adherence of either PhA1 or PhA2 to BT cells was greater than to MDBK cells (Fig 4).

We were able to identify significant differences in percentage of adherence between single strains of PhA1 and PhA2. This indicated that the fluorometric assay had sufficient sensitivity and precision (Shah *et al*, 1992) (coefficient of variation < 15% ) to discriminate between treatment effects even when responses were small (eg, < 5% adherence).

Effect of PhA1 Capsule on Adherence

It was demonstrated that the presence of a capsule on *P. haemolytica* decreased adherence of both PhA1 and PhA2 to bovine cells. Both PhA1 and PhA2 adherence was

significantly greater at 18 hours on both MDBK and BT cells (p<0.01) (Figs. 5 and 6). To check that this adherence was due largely to capsule, a 6 hour culture of PhA1 was stripped of capsule and compared with 6 hour capsulated adherence. Again, non-capsulated bacteria showed significantly greater adherence (p<0.01) (Fig. 7).

## Effect of Blocking with Sarkosyl-derived OMPs on Adherence

Although not significant, there appeared to be some blocking of adherence of PhA1 to both MDBK and BT cells, whilst PhA2 adherence remained unaffected (Figs. 8 and 9). Differing fom previous results, there appeared to be no significant difference in adherence of both A1 and A2 isolates to both cell types, and adherence appeared to be greater on MDBK than BT cells.

## Discussion

The *in vitro* fluorometric assay described in the present study is a time-efficient, inexpensive, and labor-saving method for evaluation of *P. haemolytica* adherence. Adherence could be measured after a 45 minute incubation period, thus avoiding potential variability in results associated with continued bacterial replication. In contrast to the fluorometric assay, quantitative culture requires additional steps, such as release of adherent bacteria from cells by addition of detergent and subsequent bacteriologic culture of lysate dilutions. These additional steps may contribute to experimental error.

The fluorometric assay may be particularly useful for evaluating the possible role of serovar-specific bacterial virulence factors on adherence. It can be argued that neither

MDBK nor BT cells are ideal for the study of bacterial adherence to bovine respiratory epithelium. Madin Darby bovine kidney cells are epithelial in type but are not derived from upper respiratory tract tissue, whereas BT cells are derived from the nasopharynx but are fibroblastic in type. However, there are few established bovine or other ruminant epithelial cell lines available for use. Despite the low percentage of adherence ($< 5\%$) determined for both PhA1 and PhA2 to MDBK and BT cells, these established cell lines were adequate for comparative analysis of adherence. Ideally, the bovine respiratory tract would best be represented *in vitro* by primary cell cultures that mimic the air-liquid interface of this mucosa, but the labor and difficulty involved in obtaining these types of cells, their limited life-span *in vitro*, and the inter-assay variability associated with the use of primary cell cultures complicates their use in assays such as that described in the present study.

We found that a single strain of PhA1 and PhA2 did adhere to both MDBK and BT cells, which suggests that adherence may constitute an important stage in the early pathogenesis of BRD. To our knowledge, this is the first report that describes results of *in vitro* experiments examining adherence of *P. haemolytica* to mammalian cells. However, adherence of many other important pathogens of the *Pasteurellaceae* family, such as *Haemophilus influenzae* and *Actinobacillus pleuropneumoniae*, have been examined (van Alphen *et al*, 1996; St. Geme and Falkow, 1990; Jacques *et al*, 1991). Generally, adherence of *Pasteurellaceae* organisms is influenced by a variety of bacterial virulence factors, including capsule, lipopolysaccharide, fimbriae, and outer membrane proteins. Irrespective of whether these factors promote specific or nonspecific interactions between host cells and bacteria, it is likely that variation in expression of

specific virulence factors among bacterial strains or serovars within the same species correlates with differences in adherence. Indeed, the lack of capsule or the presence of fimbriae is known to promote adherence of *Haemophilus influenzae* to human respiratory epithelium (van Alphen *et al*, 1996; St. Geme and Falkow, 1990; Bakaletz *et al*, 1988). Likewise, the significant difference in percentage of adherence that we detected between a single strain of PhA1 and PhA2 may be related to serovar-specific expression of bacterial adhesins, which have yet to be definitively identified and studied.

The greater percentage of adherence of PhA1 to BT cells, compared with PhA2, suggests that the predominance of PhA1 over PhA2 during the early stages of BRD may be attributable to the ability of PhA1 to adhere more avidly to nasopharyngeal tissues. Increased adherence would provide protection against mucociliary and other clearance mechanisms, and promote the rapid replication required to yield a large enough concentration of bacteria to allow for colonization of the lower respiratory tract. However, the present study compared adherences of only single strains of PhA1 and PhA2; further studies that compare many strains of each serovar are necessary before definitive conclusions regarding the relative adherence of each serovar can be made.

One of the reasons for development of the *in vitro* fluorometric assay was to examine the role of potential *P. haemolytica* adhesins, namely, capsule and outer membrane proteins (OMPs), on adherence to bovine respiratory and epithelial cells. Unfortunately, the fluorometric assay could not be used to study the effect of the presence or absence of capsule on PhA1 and PhA2, as capsulated bacteria showed decreased and inconsistent labeling when compared to noncapsulated bacteria. The decrease in labeling of capsulated bacteria was probably due to decreased exposure of

surface amines to which FITC binds. However, adherence was comparable on both MDBK and BT cells using direct quantitative culturing. Using this method, it was found that noncapsulated PhA1 and PhA2 adhered to both MDBK and BT cells significantly more than capsulated bacteria, indicating that capsule did not play a role in adherence in this *in vitro* situation. Indeed, it may have blocked potential bacterial surface adhesins, which seemed to be exposed and available for adherence on the decapsulated bacteria. It is unknown whether *P. haemolytica* capsular material plays a role in *in vivo* adherence, and if so, whether that adherence is specific or nonspecific to the epithelial surface or to mucus. Uhlich *et al* (1993) and Botcher *et al* (1993) have found that PhA1 adheres to bovine nasal mucus and tracheal mucus, respectively. However, in this *in vitro* assay, mucus was not present to test for any bacterial adherence.

Results of the effect of blocking of PhA1 and PhA2 adherence with OMPs, were inconclusive. Although not significant, there appeared to be some blocking of PhA1 on both cell types, indicating that there were perhaps certain adhesins on the surface of PhA1 that played a role in adherence, that were not present on the surface of PhA2. However, the blocking experiment results were difficult to interpret because of the very low and similar mean % adherences for both PhA1 and PhA2, when compared to the previous study comparing PhA1 and PhA2 adherence.

Thus, despite difficulties encountered in studying the effect of capsule and OMPs in these experiments, the availability of an *in vitro* fluorometric assay should facilitate further study of various strains of PhA1 and PhA2 and allow for screening of other specific factors that influence adherence of PhA1 and PhA2 to bovine epithelium.
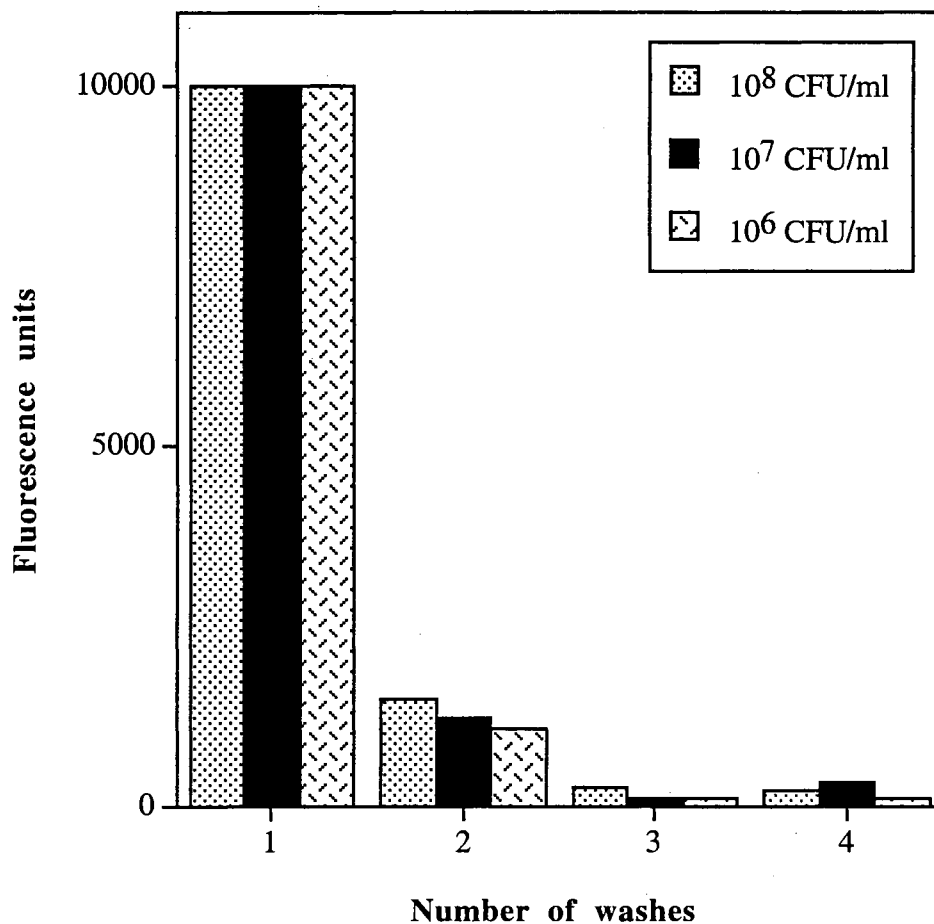
Figure 1. Amount of fluorescence (fluorescence units) remaining in supernatant of bacterial pellets after sequential washes to remove unbound fluorescein-5-isothiocyanate (FITC). *Pasteurella haemolytica* serovar A1 (PhA1) strain 668 was incubated with FITC for 1 hour at 4 C, diluted to 1 x $10^6$, 1 x $10^7$, and 1 x $10^8$ colony-forming units (CFU)/ml (based on optical density (OD) at 650 nm), and washed 3 times with 10 ml of phosphate buffered saline solution. Fluorescence in supernatant was determined with a spectrofluorometer. Fluorescence units represent scaled instrument response values; 10,000 fluorescent units was the maximum reading.
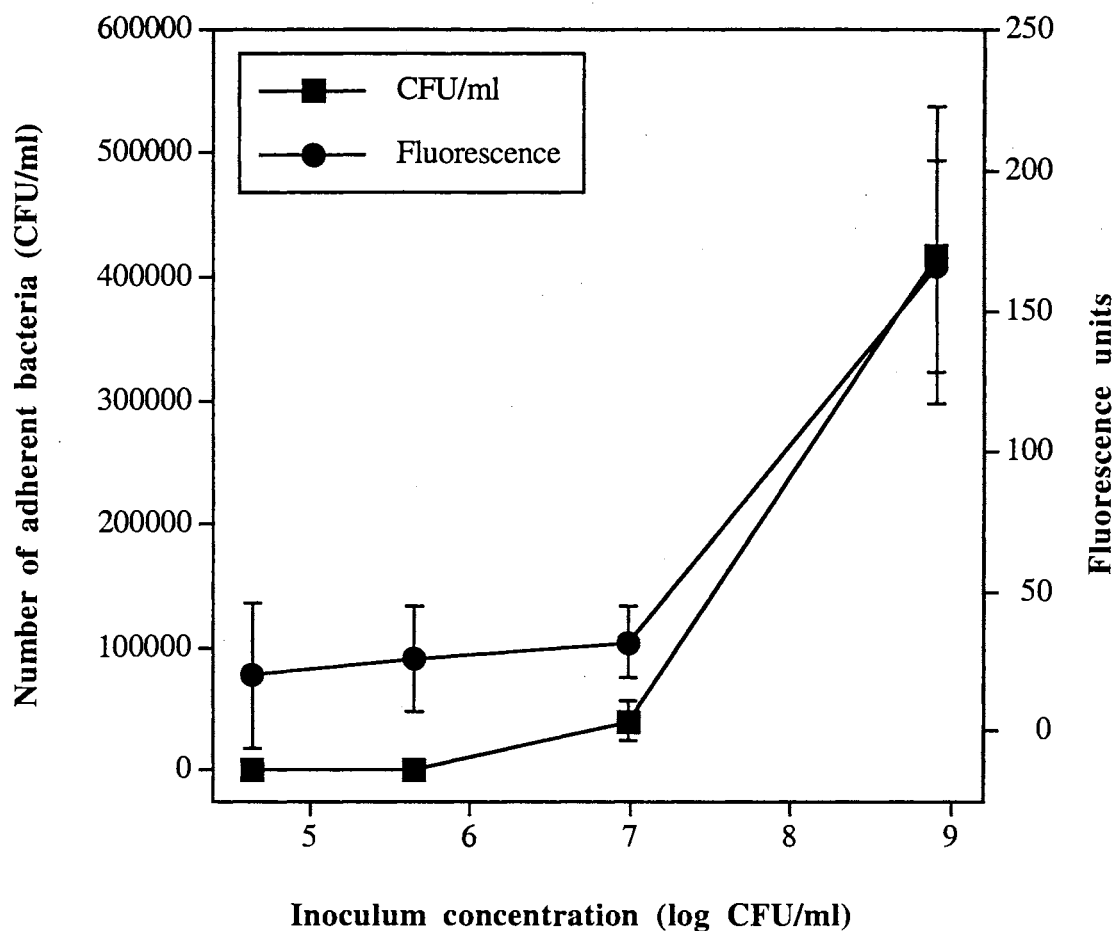
Figure 2.  Effect of concentration of FITC-labeled PhA1 strain 668 (5 x $10^4$, 5 x $10^5$, 1 x $10^{7,}$ and 8 x $10^8$ CFU/ml) added to monolayers of Madin Darby bovine kidney (MDBK) cells, on adherence.  Fluorescein-labeled bacteria were incubated with MDBK cells for 45 minutes at 37 C in 5% $CO_2$, cell monolayers were washed 5 times to remove nonadhered bacteria, and number of adherent bacteria was estimated fluorometrically (fluorescence) and by use of a quantitative bacteriologic culture technique (CFU/ml).  Each point represents the mean value for 3 wells; bars represent SD.
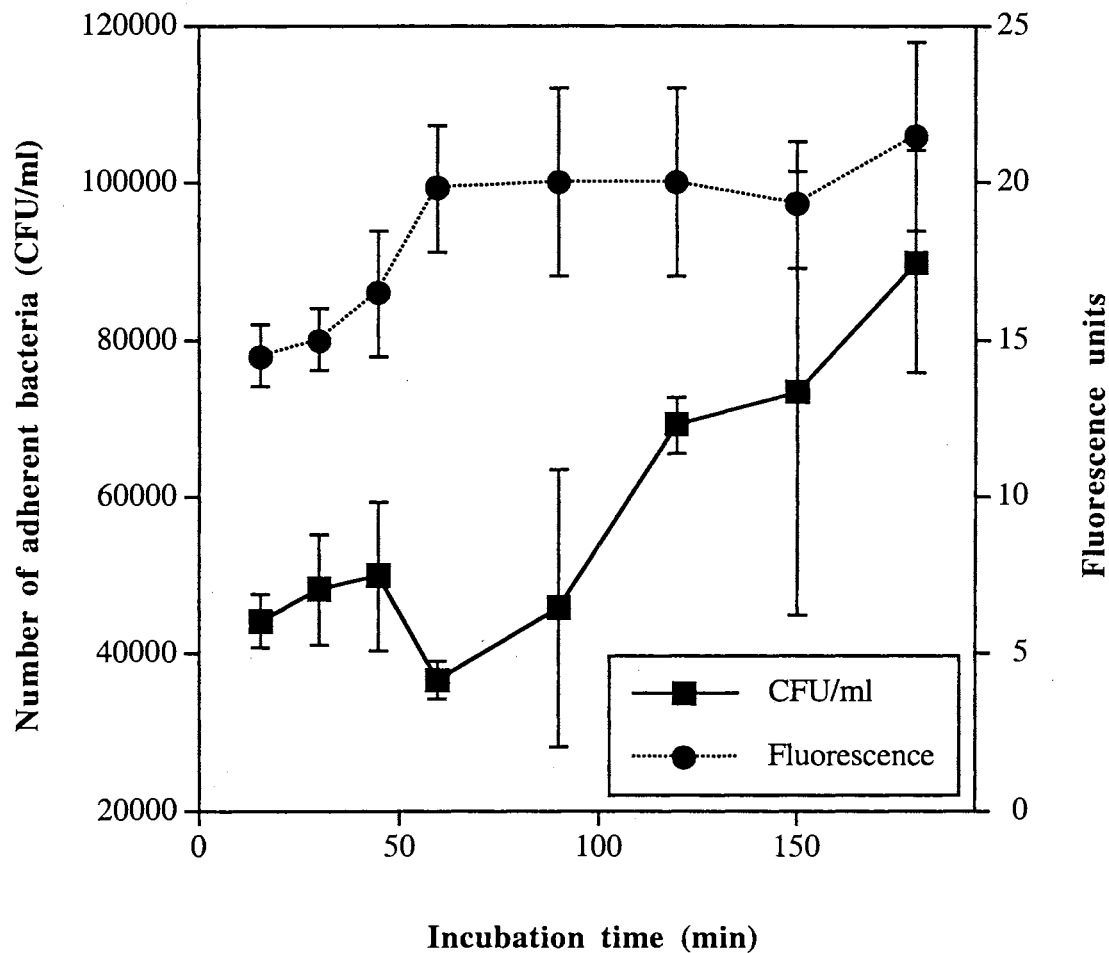
Figure 3. Effect of incubation period (15, 30, 45, 60, 90, 120, 150, and 180 minutes) on adherence of FITC-labeled PhA1 strain 668 ($1 \times 10^8$ CFU/ml) to MDBK cells. Each point represents the mean value for 6 wells; bars represent SD. *See* Figure 2 for key.
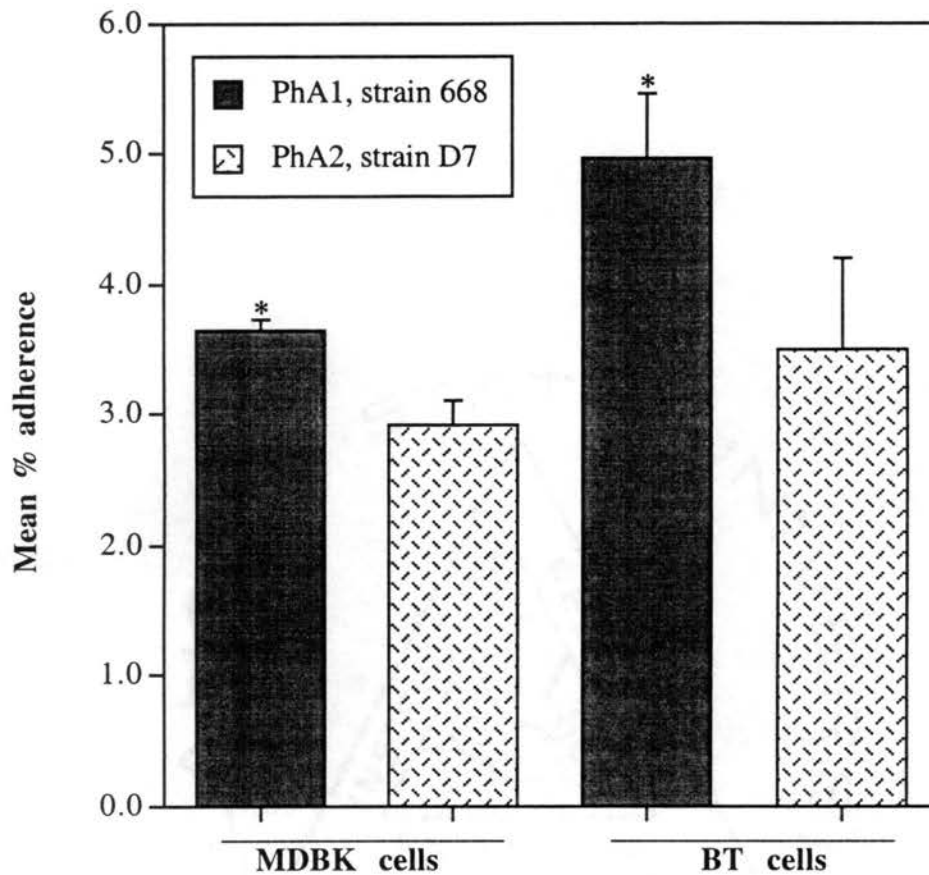
Figure 4. Mean (± SD) percentage of adherence of PhA1 strain 668 and *P haemolytica* serovar A2 (PhA2) strain D7 to MDBK and bovine turbinate (BT) cell monolayers. Monolayers were inoculated with 50 μl of FITC-labeled PhA1 or PhA2 (1 x $10^8$ CFU/ml) per well (n = 5 wells/group) and incubated for 45 minutes at 37 C in 5% $CO_2$. Duplicate sets of wells were inoculated with labeled bacteria so that, after incubation, 1 set of wells was washed 5 times to remove nonadherent bacteria and 1 set was not washed. Fluorescence in washed wells and unwashed wells was determined with a spectrofluorometer, and percentage of adherence was calculated (% adherence = [fluorescence in washed wells/fluorescence in unwashed wells] X 100). *Significantly ($P <$ 0.05) more than percentage of adherence of PhA1 to the same cell line.
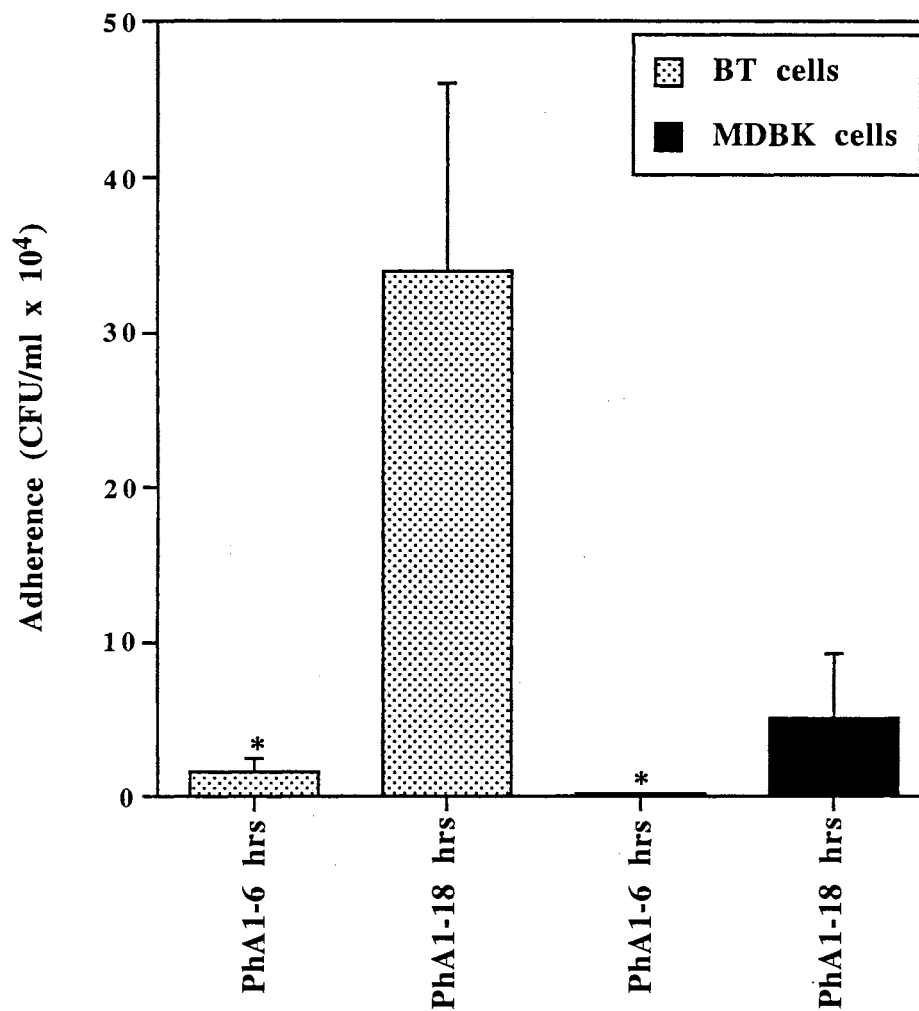
Figure 5. Average adherence (x $10^4$ CFU/ml) to MDBK and BT cells of *P. haemolytica* A1 strain 668 grown on a blood agar plate for 6 and 18 hours. * Significantly (P<0.01) less than adherence of PhA1 after 18 hours incubation on both cell lines.
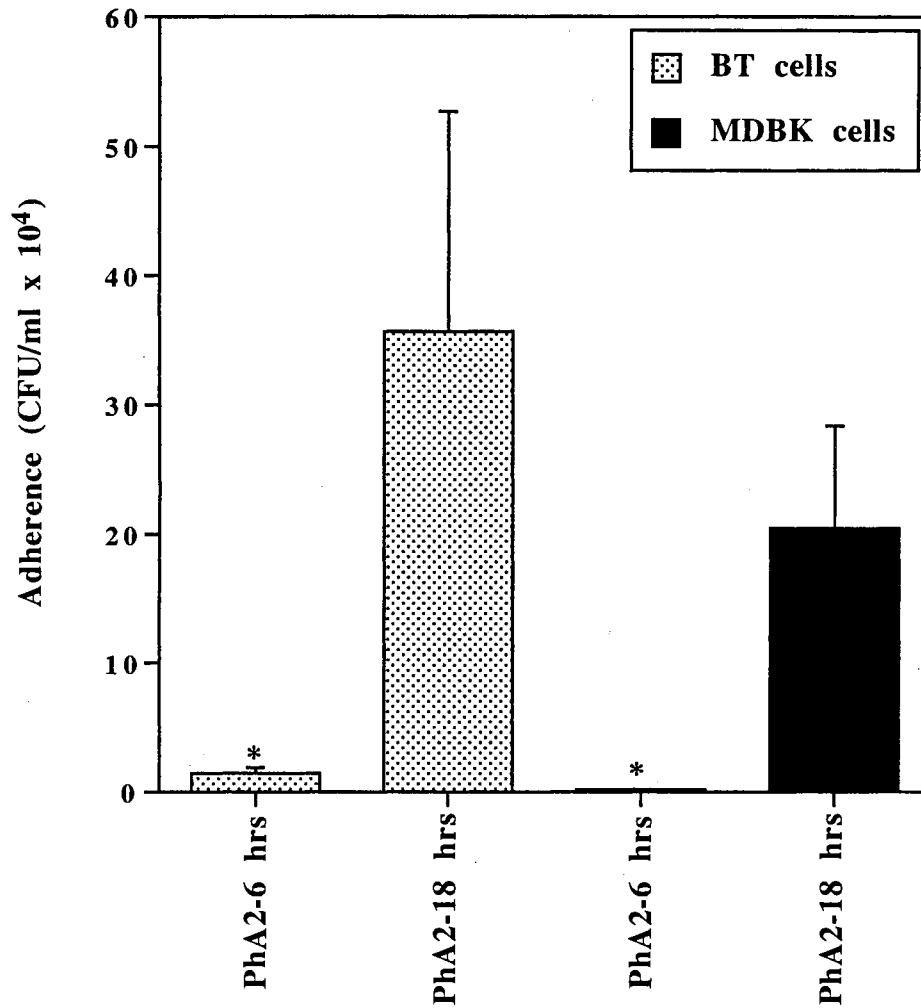
Figure 6. Average adherence (x $10^4$ CFU/ml) to MDBK and BT cells of *P. haemolytica* A2 strain D7 grown on blood agar plates for 6 and 18 hours. * Significantly (P<0.01) less than adherence of PhA2 after 18 hours incubation on both cell lines.
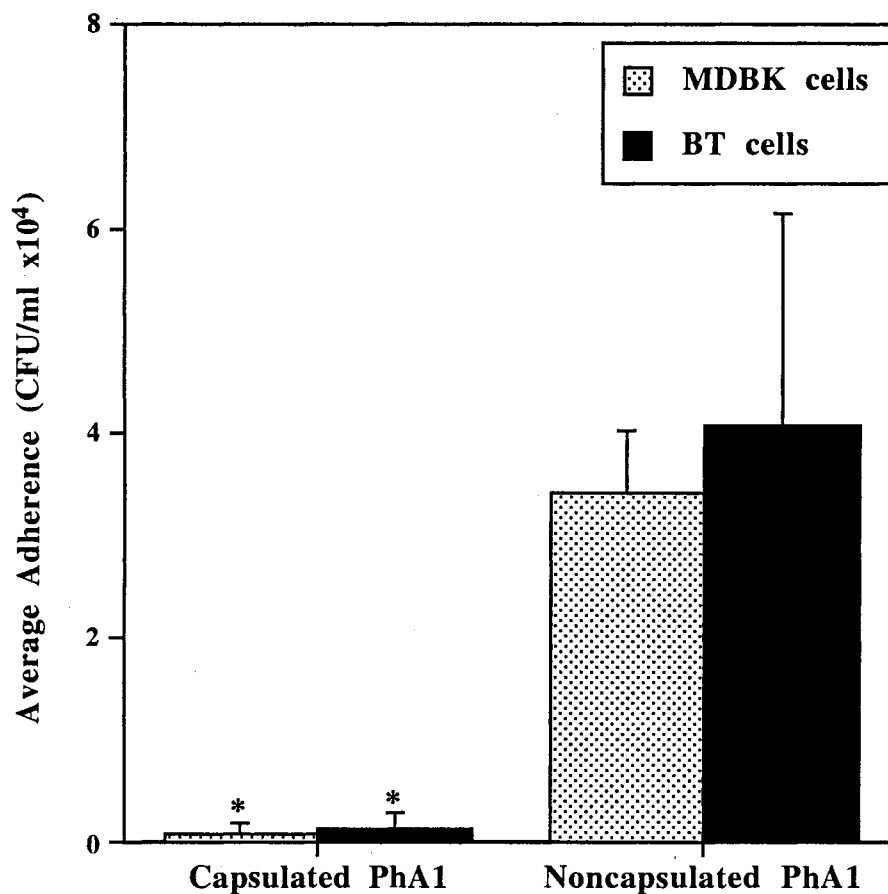
Figure 7.  Average adherence (x $10^4$ CFU/ml) to MDBK and BT cells of a 6-hour culture of capsulated and decapsulated *P. haemolytica* A1 strain 668. * Significantly (P<0.01) less than adherence of noncapsulated bacteria to both cell lines.
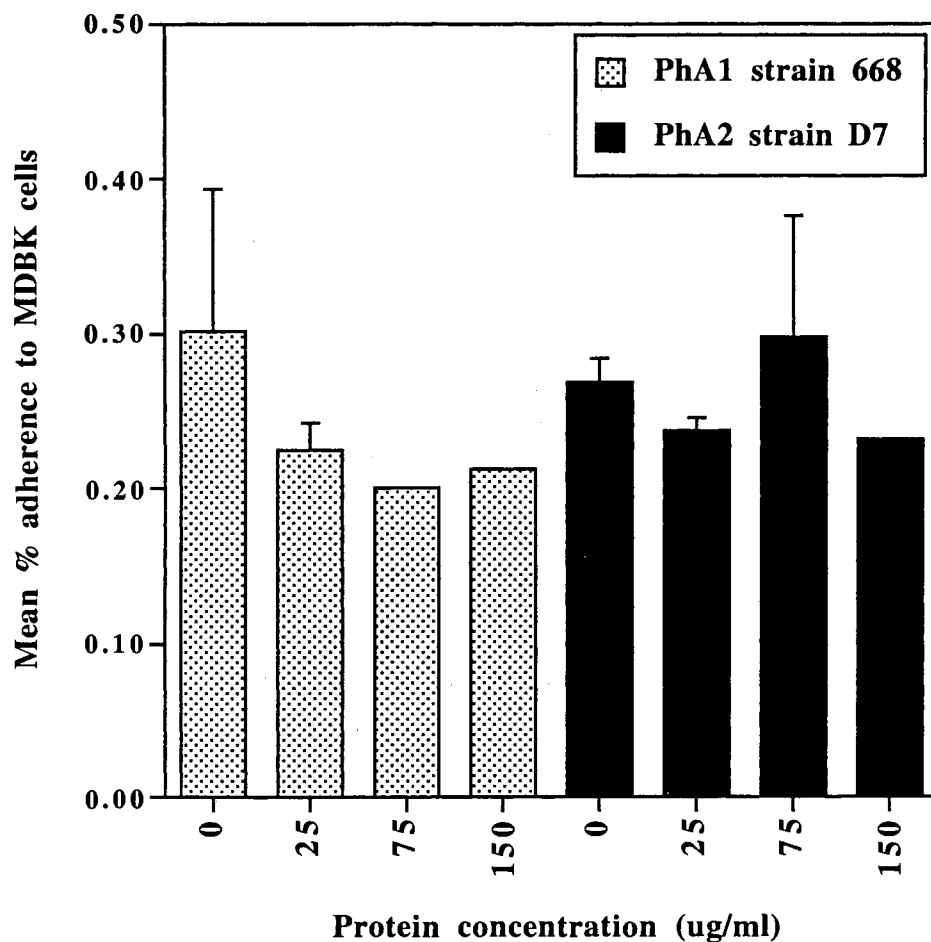
Figure 8. Mean % adherence of *P. haemolytica* A1 strain 668 and *P. haemolytica* A2 strain D7 to MDBK cells preincubated for 1 hour at 37C with various concentrations of PhA1 and PhA2 OMP preparations respectively.
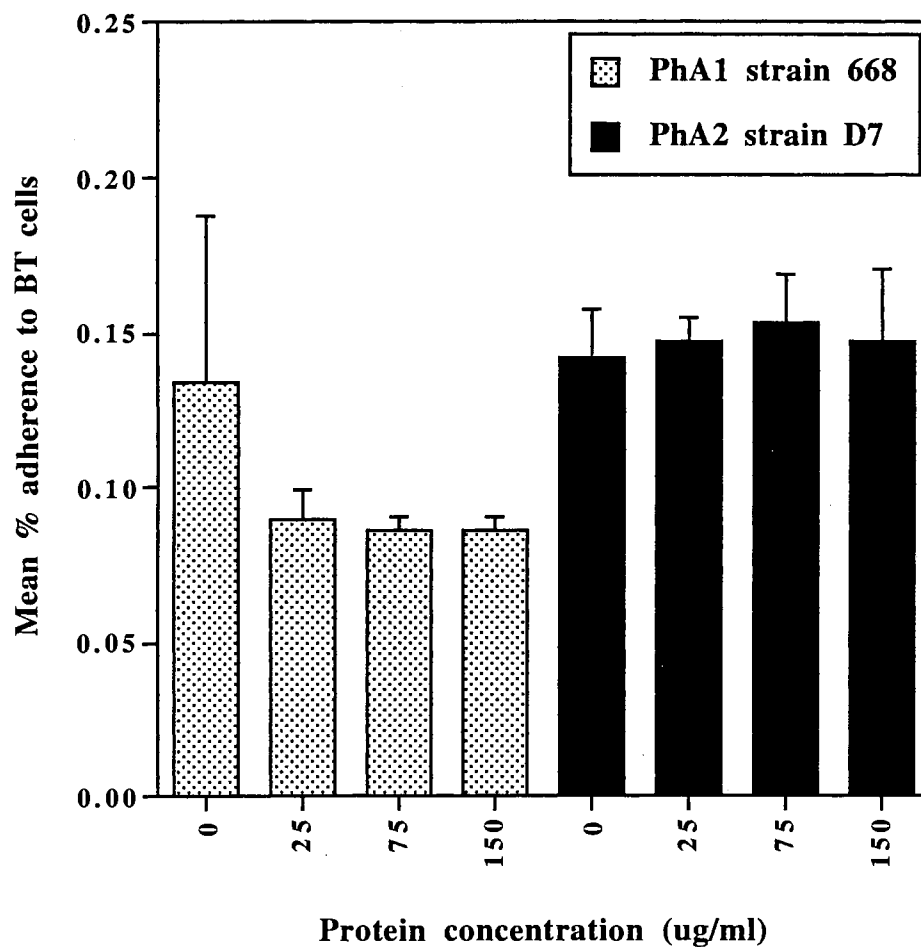
Figure 9. Mean % adherence of *P. haemolytica* A1 strain 668 and *P. haemolytica* A2 strain D7 to BT cells preincubated for 1 hour at 37C with various concentrations of PhA1 and PhA2 OMP preparations respectively.

CHAPTER V

DEVELOPMENT OF AN *EX VIVO* MODEL TO STUDY ADHERENCE OF

*PASTEURELLA HAEMOLYTICA* SEROVAR A1 TO MUCOSAL TISSUES OF THE

UPPER RESPIRATORY TRACT OF CATTLE

Introduction

Bovine pneumonic pasteurellosis or shipping fever causes considerable economic loss to the beef cattle industry, particularly to stocker and feedlot operations. *Pasteurella haemolytica* serovar A1 is isolated with greater frequency than other serovars from the lungs of pneumonic cattle (Wray and Thompson, 1971; Frank and Smith, 1983; Purdy *et al*, 1997) and is believed to be the principal cause of pneumonic pasteurellosis. Whether serovar A1 is carried in the nasopharynx and/or tonsils of healthy animals or is acquired from other diseased animals, stress and/or viral infection (Frank, 1986) are believed to promote rapid multiplication of *P. haemolytica* serovar A1 in the upper respiratory tract (Frank *et al*, 1986; Thomson *et al*, 1969; Thomson *et al*, 1975), resulting in inhalation of aerosolized bacteria and consequent pulmonary infection (Grey and Thomson, 1971; Jericho *et al*, 1986).

Rapid multiplication of pathogenic bacteria in the respiratory tract usually is facilitated by adherence to mucosal surfaces (Beachey, 1981); adhered bacteria are less vulnerable to expulsion by the mucociliary defense mechanism. A previous study employing *in vitro* cell cultures confirmed the ability of *P. haemolytica* to adhere to

bovine respiratory and epithelial tissues and suggested that the pathogenicity of serovar A1 was related to its greater degree of adherence to mucosal surfaces than serovar A2 (Clarke and Morton, 2000), which is more frequently isolated from healthy cattle (Frank and Smith, 1983; Purdy *et al*, 1986). However, interpretation of these studies was complicated by the methodological problems associated with the failure of *in vitro* cultured cell monolayers to represent the structural and functional integrity of mucosal components such as ciliated epithelium and mucus. These types of models (St. Geme and Falkow, 1990; St. Geme and Cutter, 1995), as well as other *in vitro* methods such as those employing epithelial cell suspensions (Glorioso *et al*, 1982; St. Geme and Cutter 1996) and tracheal explants (Dugal *et al*, 1992; Bélanger *et al*, 1990), do not adequately mimic bovine upper respiratory epitheial cells *in situ* and have inherent problems that include adherence of bacteria to surfaces other than host cells and difficulty in measuring or enumerating adherent bacteria. *In vivo* field studies (Jericho *et al*, 1976; Friend *et al*, 1977a; Friend *et al*, 1977b; Frank, 1988) have provided important information on the epidemiology of the disease, but because of the logistics and expense of working with large numbers of cattle, and the complexity of the upper respiratory mucosal environment, they do not provide a means for studying specific interactions between bacteria and the bovine host cells at the cellular and molecular level.

Therefore, the purpose of this study was to develop and validate an *ex vivo* model for study of adherence of *P. haemolytica* to upper respiratory tract mucosa of cattle and to use this model to confirm adherence of *P. haemolytica* serovar A1 to several relevant respiratory mucosal surfaces. It is expected that the availability of this model will

facilitate investigation of the early pathogenesis of pneumonic pasteurellosis and development of effective strategies for management of the disease.

Materials and Methods

Radiolabeling of Bacteria

*Pasteurella haemolytica* A1 strain 668 (PhA1), a low passage strain isolated from the lung of a calf with pneumonic pasteurellosis (obtained from the Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK), was cultured for 18 hours on blood agar before bacteria were harvested in 3 ml leucine-deficient Roswell Park Memorial Institute media (ICN Biomedicals, Inc.) plus 1 ug/ml L-glutamine (JRH Biosciences) (leucine-deficient RPMI). The harvest was diluted with leucine-deficient RPMI to an optical density at 600nm ($OD_{600nm}$) of approximately 0.120 using a spectrophotometer (Turner Model 690 Spectrophotometer, Barnstead/Thermolyne), and incubated in an incubator shaker (Innova 4000 Incubator Shaker, New Brunswick Scientific Co., Inc.) at 37C and 120 oscillations/minute for approximately 4 hours or until midlog phase was reached ($OD_{600nm}$ = 0.530, estimated from a previously constructed growth curve for PhA1). Colony-forming units/ml (CFU/ml) were determined using a standard curve and the bacterial concentration was confirmed by overnight spot-plate counts using the Miles-Misra technique (Quinn *et al*, 1994).

Suspended bacteria were incubated with 2 µCi/ml of tritiated leucine ($[^3H]$-leucine; ICN Pharmaceuticals, Inc.) for 1 hour at 37C, with occasional shaking. Radiolabeled bacteria were then centrifuged (Sorvall RC2-B, rotor SM-24) at 8000 rpm for 10 minutes

and the pellet washed by centrifuging three times in sterile phosphate buffered saline (PBS) to remove any unincorporated [$^3$H]-leucine. The pellet was then resuspended in PBS and refrigerated at 4C overnight, after which the radiolabeled inoculum was washed once by centrifugation and resuspended in PBS to the desired bacterial concentration, determined spectrophotometrically and confirmed using spot-plate counts. Radioactivity was measured in liquid scintillant (Atom-Light; Packard Instrument Co., Inc.) using a liquid scintillation counter (Model LS 5000TD, Beckman Instruments Inc.). Preliminary experiments confirmed that the radiolabeling procedure provided easily measurable levels of radioactivity after repeated washing and dilution of the inoculum, that bacterial viability was maintained with overnight refrigeration, and that four washes removed almost all excess radiolabel not incorporated into the bacteria.

Preparation of Mucosal Tissue

Mucosal tissues were collected from freshly slaughtered steers after resecting and exposing the nasal cavity, turbinates, and pharynx. Prior to tissue collection, an area immediately adjacent to each of the chosen tissue sites was swabbed and the sample was cultured on blood agar at 37C overnight to test for microbial contamination. Sections (2 x 2 cm or greater) of nasal, dorsal turbinate, nasopharyngeal, and palatine tonsillar mucosa were resected and examined by light microscopy to confirm the presence of smooth undamaged tissue surfaces, mucus production, and beating cilia, where applicable. Tissue sections were placed in RPMI containing antibiotics (20 µg/ml gentamicin [Sigma Chemical Co.] and 20 µg/ml sodium ampicillin [Marsam Pharmaceutical, Inc.]) and incubated at 37C with occasional agitation for 1 hour.

Thereafter, tissues were washed in antibiotic-free RPMI before equlibrating in antibiotic-free media for 1 hour at 37C, with occasional agitation. Using a 6 mm diameter biopsy punch, small tissue samples were prepared and placed in fresh antibiotic-free RPMI at 37C. Sterility of tissue preparation was confirmed by culture of random samples on blood agar. Tissue samples were then embedded, adventitial surface upwards, in 15 ul molten 1% agarose contained in wells of 96-well tissue culture plates (Corning Glass Works). The agar was allowed to cool and solidify, thus preventing bacterial adherence to the underside and sides of the tissue samples (Fig. 1).

## Adherence Assay

Adherences to tissue samples were tested by pipetting 50 $\mu$l of radiolabeled inoculum or PBS into treatment and control wells, respectively. After incubating for the required times at 37C, nonadherent bacteria were removed by washing embedded tissue samples 5x with sterile PBS using an automatic washer (Nunc Immuno-Wash 12, Nunc Brand Products). Tissue samples were then removed from the wells, residual agar was removed from the underside and sides of the tissues, and each sample was placed in a 5 ml scintillation vial containing 0.5 ml Soluene-350 (Packard Instrument Co.) for overnight digestion. Radioactivity of digested samples was measured in Atom-Light liquid scintillant using a liquid scintillation counter (Model LS 5000TD, Beckman Instruments, Inc.). Radiolabeled inocula were also subjected to direct liquid scintillation counting to obtain the total radioactivity of bacteria added to the tissue samples.

## Model Validation

Optimal inoculation concentration and incubation time were determined by measuring adherences after incubation of several PhA1 inoculum concentrations ($1 \times 10^8$, $1 \times 10^7$, and $1 \times 10^6$ CFU/ml) on nasopharyngeal tissue samples for 1, 3, 6, and 18 hours (n = 4). Data comparing adherences of the different PhA1 concentrations and incubation times were analyzed using a 3 x 4 factorial arrangement of treatments. Significance was declared at the $P < 0.05$ level. Based on these experiments, an inoculum concentration of $1 \times 10^7$ CFU/ml and an incubation time of 3 hours were employed for all further experiments.

Light microscopy of tissue samples fixed in Bouin's fixative and stained with hematoxylin-eosin was conducted to confirm that the samples were both representative of the intended region of study and had intact and healthy surfaces. Samples subjected to these examinations included control tissues as well as tissues incubated with bacteria for 3 or 18 hours.

Scanning (SEM) and transmission (TEM) electron microscopy were used qualitatively to confirm adherence of *P. haemolytica* to mucosal tissues and to identify interactions between bacteria and specific components of epithelial tissues, such as cell membranes, cilia, and mucus. Adherence assays were performed as described above, after which nasopharyngeal tissue samples were fixed in 3% cacodylate-buffered glutaraldehyde for 2 hours at 4C, followed by 3, 20-minute 0.1M cacodylate buffer washes. Tissues were post-fixed in 1% cacodylate-buffered osmium tetroxide ($OsO_4$) for 2 hours at 4C, washed again, and left refrigerated overnight in the third wash. After dehydration in a graded series of ethanol, tissues were cleared in propylene oxide,

infiltrated, embedded in Poly/Bed 812 (Polysciences, Inc.) and polymerized for 3 days at 60C. Thin sections were cut and stained with lead citrate and uranyl acetate, and tissue was examined using a transmission electron microscope (JEOL 100 CX-11 STEM). For SEM, dehydrated tissue was critical point dried and coated with gold/palladium. Samples were viewed using a scanning electron microscope (JEOL JSM 35U) operated at 25 kV.

The effect of antibiotic pretreatment of tissue sections on PhA1 viability and adherence was investigated by comparison between antibiotic-pretreated and nontreated nasopharyngeal tissues. To test whether antibiotic pretreatment affected viability of PhA1, replicates (n = 3) of antibiotic-pretreated and nontreated nasopharyngeal tissues were incubated with 50 μl radiolabeled PhA1, after which tissue and inoculum were harvested from each well, vortexed to dislodge and suspend bacteria, and the supernatants removed and divided into pairs of equal aliquots. Radioactivity of one aliquot was measured and the other aliquot was used for determination of CFU/ml. The effect of antibiotic pretreatment on adherence was investigated by incubating PhA1 with replicates (n = 3) of antibiotic-pretreated and nontreated nasopharyngeal tissues and then measuring the degree of adherence. Data were analyzed using unpaired t-tests. Significance was declared at the $P < 0.05$ level. Bovine nasopharyngeal tissue after antibiotic pretreatment but prior to addition of PhA1 inoculum was also viewed by scanning electron microscopy to confirm the absence of bacteria (Fig. 2).

Intra- and inter-assay precisions of the assay were estimated by measuring adherences of PhA1 to 5 replicates of nasopharyngeal tissue in 5 separate experiments. These validation parameters were expressed as coefficients of variance (%CV).

To confirm that adherence of PhA1 to nasopharyngeal tissue was specific and not merely non-specific attachment due to electrostatic forces or entrapment in tissue folds or mucus, adherence of PhA1 was compared with that of *Salmonella pullorum* (n = 4; bacteria obtained from The Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK), a gram-negative rod specific for poultry rather than ruminant tissue. Conditions and procedures for radiolabeling and assay of *Salmonella pullorum* adherence were identical to those used with PhA1. Adherences of the two types of bacteria were compared using paired t tests at the P < 0.05 level.

Adherence of *P. haemolytica* to Different Tissues of the Upper Respiratory Tract

Replicates (n = 5) of nasal, nasopharyngeal, turbinate, and palatine tonsillar tissue were incubated with 1 x $10^7$ CFU/ml PhA1 for 3 hours and adherences were measured. The experiment was repeated twice. Adherences of PhA1 to the different tissues were compared using the general linear model with repeated experiments represented as blocks. Individual means were separated using Scheffe's Test. Significance was declared at the P<0.05 level.

Results

Adherence of PhA1 to bovine nasopharyngeal tissue varied from 3.03 ± 1.54 to 33.3 ± 10.36 %, depending on the concentration of the inoculum and the incubation time (Fig. 3). Adherences after 18 hours of incubation were significantly higher than those measured at shorter incubation times. Likewise, an inoculum concentration of 1 x $10^6$

CFU/ml resulted in higher adherences than those following inoculation of higher bacterial concentrations.

Light microscopy of nasopharyngeal tissue before addition of the bacterial inoculum revealed the presence of a healthy, intact mucosal surface with cilia (Fig. 4a). The morphological integrity of the epithelial surface was not altered by incubation with media for 3 and 18 hours, nor by incubation with PhA1 for 3 hours (Fig. 4b). However, after 18 hours incubation with PhA1, there was evidence of damage to surface epithelial cells, suggested by the presence of necrotic cells, cells with apototic nuclei, and localized loss of cilia (Fig. 4c).

Examination by SEM confirmed the presence of bacteria attached to the mucosal surface, particularly where cilia were in abundance (Fig. 5). In those areas where cells were sparsely ciliated, bacteria appeared to follow the regions of ciliation and adhered to each other to form aggregations of bacteria (Fig. 6). Few bacteria were seen attached to areas covered with mucus strands (Fig. 7). Bacteria adhered to nasopharyngeal epithelium were more difficult to find on TEM than SEM. Those that were present on TEM examination were not seen attached directly to or near the epithelial surface, but appeared to be present near the tips of the longer cilia in the residual mucus layer (Fig.8). A few bacteria appeared to be attached to mucus (Fig. 9), and several were adhered to cilia (Figs. 10, 11, and 12).

Culture of samples taken from tissues before they were treated with antibiotics revealed the presence of contaminating bacteria, including *Escherichia coli* and *Staphylococcus* species, which were isolated from tonsillar epithelium, and *Pasteurella multocida, Pasteurella haemolytica, Pasteurella* species, *Haemophilus somnus,*

*Arcanobacterium pyogenes, Staphylococcus* species, and *Actinobacillus ligniersii*. Mean radioactivity and viability of PhA1 recovered from antibiotic-pretreated ($16303 \pm 2302$ cpm; $3.75 \times 10^5$ CFU/ml) and untreated ($16989 \pm 810$ cpm; $3.67 \times 10^5$ CFU/ml) tissues (Fig. 13) were not significantly different from each other, indicating that antibiotic pretreatment of nasopharyngeal tissues to remove contaminating bacteria had very little observable effect on the viability of the PhA1 inoculum. However, adherence of PhA1 to nasopharyngeal tissues pretreated with antibiotics ($12.1 \pm 0.8$ %) was significantly higher with less variability than adherence to untreated tissues ($6.4 \pm 2.3$ %), suggesting that the presence of a variable contaminating bacterial population in tissues not treated with antibiotics interfered with adherence of PhA1 (Fig. 14).

Coefficients of variation for intra-assay (ranged from $7.36 - 21.06$ % for 5 experiments) and inter-assay ($21.77$ %) precision were acceptable levels for this type of microbiological assay. Thus, the model was sufficiently reproducible (Shah *et al*, 1992) to allow further study of host- and bacterial-dependent effects on PhA1 adherence.

Mean % adherence of PhA1 to bovine nasopharyngeal tissue ($10.06 \pm 2.03$ %) was significantly higher than that of *S. pullorum* ($5.52 \pm 0.34$ %), a bacterium with host specificity for poultry (Fig. 15). Therefore, adherence of PhA1 to bovine upper respiratory epithelium involved specific interaction with adhesins not represented in the non-specific association of bovine tissue with another gram-negative bacterium that usually colonizes another host.

Comparison of adherences to mucosal tissues harvested from different regions of the upper respiratory tract of cattle revealed similar adherences to nasopharyngeal and

nasal tissues.    Adherence to nasopharyngeal tissue was significantly greater than adherences to turbinate and tonsillar tissues (Fig. 16).

## Discussion

Colonization of mucosal tissues usually involves initial adherence of bacteria to epithelial cells followed by bacterial replication.  The present study confirmed that PhA1 adheres to URT mucosa of cattle, an observation that is consistent with the hypothesized pathogenesis of pneumonic pasteurellosis:    Previous studies have suggested that pulmonary infection results from inhalation of large numbers of bacteria generated by rapid multiplication in the URT of stressed animals.  Adherence to mucosal tissues of the URT can be expected to prevent clearance of *P. haemolytica* by the mucociliary apparatus, thereby promoting bacterial replication.

The degree of adherence observed in the present study which utilized an *ex vivo* model, was similar to that observed in a previous study employing an *in vitro* model consisting of a cell culture monolayer.  An incubation period of 45 minutes to 1 hour and an inoculation dose of 1 x $10^8$ CFU/ml demonstrated PhA1 adherences of approximately 3%, irrespective of the models employed.  However, the *ex vivo* model more closely represents the functional and structural characteristics of URT mucosa and can be expected to provide a more accurate assessment of adherence.  In particular, cell culture monolayers are relatively undifferentiated and do not have specialized structures and functions such as cilia and mucus production, which are believed to participate in bacterial adherence.  In contrast to the *in vitro* model, histologic and electron microscopic examination of the tissues used in the *ex vivo* model confirmed the structural integrity of

epithelial cells. Indeed, electron microscopy indicated the participation of epithelial cell cilia and surface mucus in adherence of bacteria, observations that would not have been possible using *in vitro* cell culture monolayers. Specific bacterial adhesins or epithelial cell surface receptors involved in PhA1 adherence have yet to be identified, but can be easily explored using the *ex vivo* model because they are likely to be expressed in differentiated URT epithelial cells. The importance of using differentiated cells in adherence studies has been demonstrated by researchers investigating adherence of other respiratory pathogens: *Streptococcus pneumoniae* adheres and replicates only in the mucus layer and never attaches to ciliated cells or cilia (Feldman *et al*, 1992) whereas *Bordetella pertussis* adheres specifically to cilia (Tuomanan, 1988).

Aside from the failure of *in vitro* cell monolayers to accurately represent the differentiated mucosal tissues involved in adherence, another relative disadvantage of *in vitro* cell monolayer models is that bacteria may attach to culture plate surfaces when growth of host cells is not entirely confluent. Furthermore, use of radiolabeled bacteria in the *ex vivo* model avoids the requirement to enumerate adhered bacteria by culture or the use of fluorescent probes to estimate proportional adherence. Culture of adhered bacteria may be complicated by the difficulty in separating them from host cells. Radioactivity can be detected with greater sensitivity than fluorescent probes, which are also subject to photobleaching and quenching, and may physically block potential binding sites on the bacterial surface.

Percent adherences of radiolabeled PhA1 to nasopharyngeal tissue varied depending on the inoculum concentration and incubation time. Although the number of bacteria adhering to tissue increased with increasing inoculum concentration, when

expressed as a proportion of total bacteria in the inoculum, the % adherence decreased with increasing inoculum concentration. This trend suggested that bacteria competed for available binding sites on the nasopharyngeal tissue and that a greater proportion of the inoculated bacteria were able to associate with these sites at lower inoculum concentrations. The increase in adherence observed at longer incubation times suggested that there was an increase in available binding sites, possibly as a result of loss of mucus and exposure of binding sites or increased likelihood that bacteria would come into contact with binding sites as cilia continued to beat. The inoculum concentration and incubation times selected for the assay (1 x $10^7$ CFU/ml and 3 hours, respectively) reflected a compromise between the need for an easily measurable response and the possibility that saturation of binding sites may occur at high inoculum concentrations and/or long incubation times.

The presence of a variety of different bacteria on freshly harvested URT mucosal tissues necessitated the elimination of these before the tissues were used in adherence assays. The possibility that these contaminants could interfere with adherence of PhA1 was confirmed by comparison between adherences to antibiotic-pretreated and untreated tissues. There was no apparent residual effect of antibiotics on the viability of the PhA1 inoculum employed in the adherence assays. The inhibitory effect of bacterial contaminants on PhA1 adherence and the observation that the proportional adherence of PhA1 decreased as the inoculum concentration increased suggest that adherence was saturable and sufficiently specific in nature to involve competition of bacteria for attachment sites. It is not known whether adherence involved interactions between bacterial adhesins and host cell receptors or bacterial capsule and mucus. Examination

by SEM demonstrated the presence of many adhered bacteria in the ciliated regions of the mucosa, suggesting that attachment to cilia may be an important mechanism of PhA1 adherence. The possible involvement of cilia was supported by TEM examination, which revealed several bacteria in close association with cilia, although no specialized structures such as fimbriae could be observed in zones of apparent attachment. In several instances, TEM examination indicated the presence of bacteria associated with mucus, indicating that adherence of PhA1 may involve attachment between bacteria and a variety of host tissue components, both specific and nonspecific. The increase in adherence observed when tissues were incubated for longer periods of time could be explained by damage to epithelial cells by bacterial secretory products such as neuraminidase which removes sialic acid from cell surface glycoproteins (Whiteley *et al*, 1992), or proteases which promote degradation of fibronectin (Woods, 1987), resulting in exposure of host cell receptors to bacterial adhesins. It is also possible that continued production of mucus by epithelial cells may have resulted in entrapment of larger numbers of bacteria.

Comparison between mucosal tissues collected from different regions of the bovine URT indicated that adherence of PhA1 to nasal and nasopharyngeal mucosa was significantly greater than to tonsillar and turbinate tissues. Data derived from use of tonsillar tissues were not easily interpreted because of the difficulty in obtaining thin, uniform sections of tissue without including underlying submucosal gland tissue that continued to secrete mucus throughout the assays. The evidence that adherence was greatest in the nasopharyngeal region is consistent with the observations of Frank and Smith (1983), who reported that rapid growth and colonization of *P. haemolytica* A1 occurred in the nasopharynx of stressed cattle recently introduced into the feedlot. The

present study provides strong evidence that this explosive growth of bacteria in the

bovine URT involves adherence to mucosal tissues.
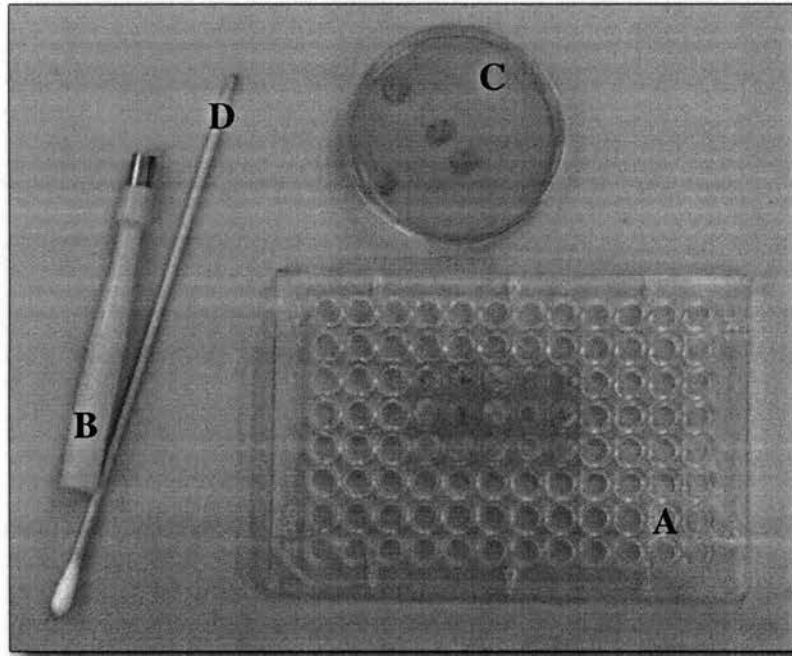
Figure 1. A 96-well tissue culture plate with samples of bovine nasopharyngeal tissue in each of fifteen wells previously layered with molten agar (A). Samples were removed from a large piece of nasopharyngeal tissue using a biopsy punch (B), placed in a petri-dish containing warmed media (C), and then lifted and placed into tissue culture plate wells using the biopsy punch and swab (D).
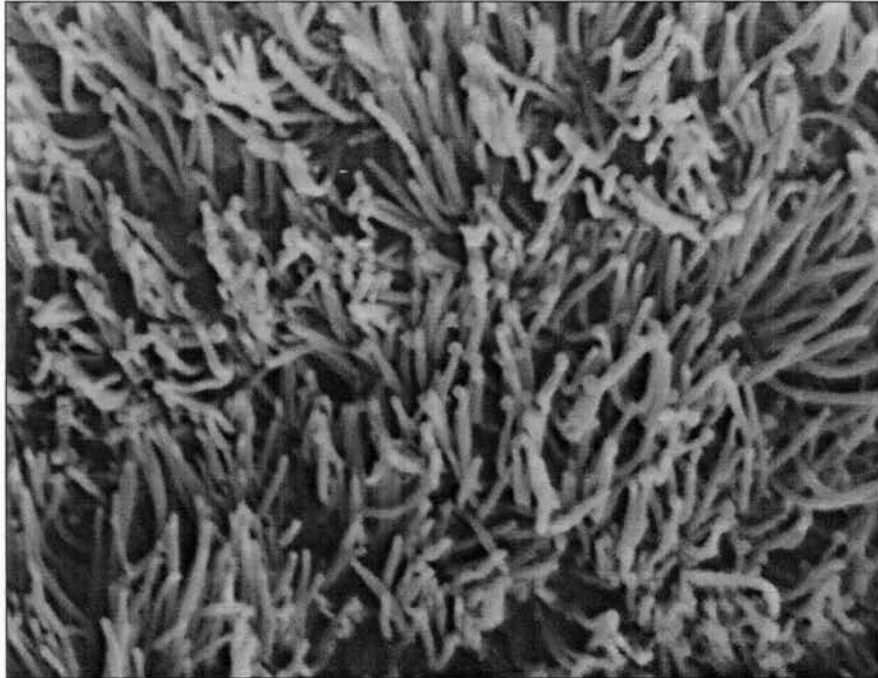
Figure 2. Scanning electron micrograph of antibiotic-pretreated bovine nasopharyngeal tissue prior to incubation with *P. haemolytica*. Note the absence of bacteria on the ciliated epithelial surface (5500 X).
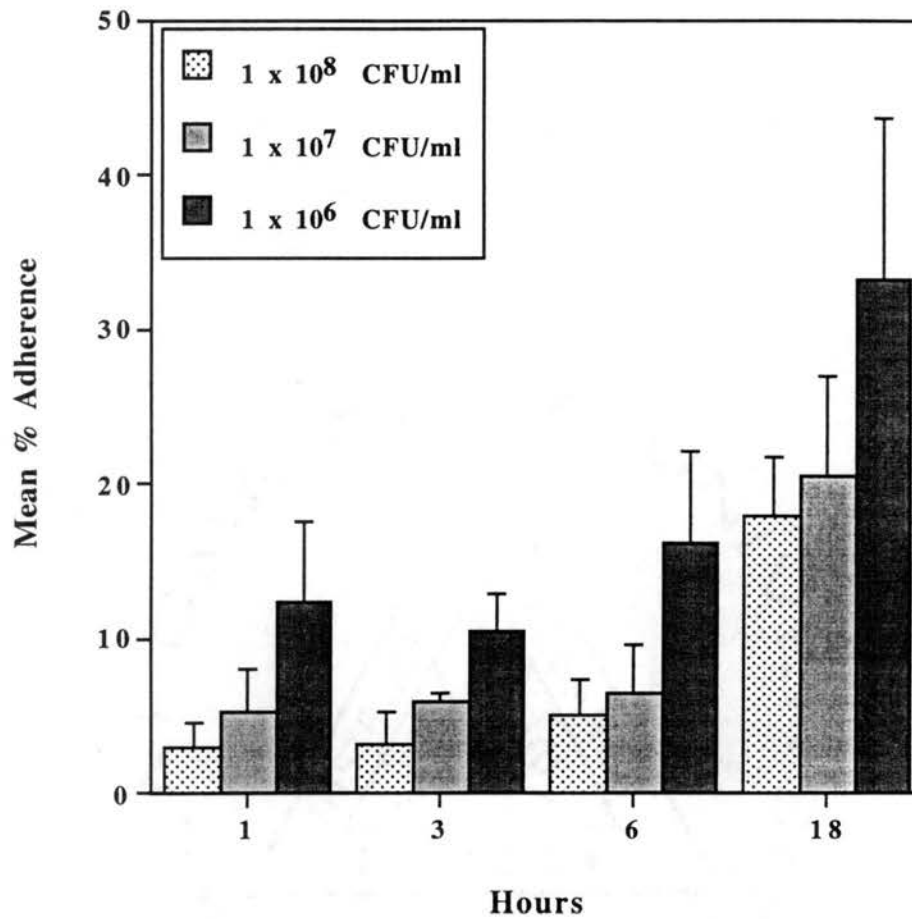
Figure 3. Effect of inoculum concentration and incubation time on adherence of *P. haemolytica* to bovine nasopharyngeal tissue (n=5). Adherence of 50 μl of each of three PhA1 inoculum concentrations ($1 \times 10^8$, $1 \times 10^7$, and $1 \times 10^6$ CFU/ml) at four incubation times (1, 3, 6, and 18 hours) was measured on antibiotic-pretreated nasopharyngeal tissue. After incubation, tissue was washed, removed, and radioactivity (Cpm) of adherent bacteria measured. Mean (±SD) adherences at 18 hours and $1 \times 10^6$ CFU/ml were significantly higher than those at shorter incubation times and inoculum concentrations, respectively.
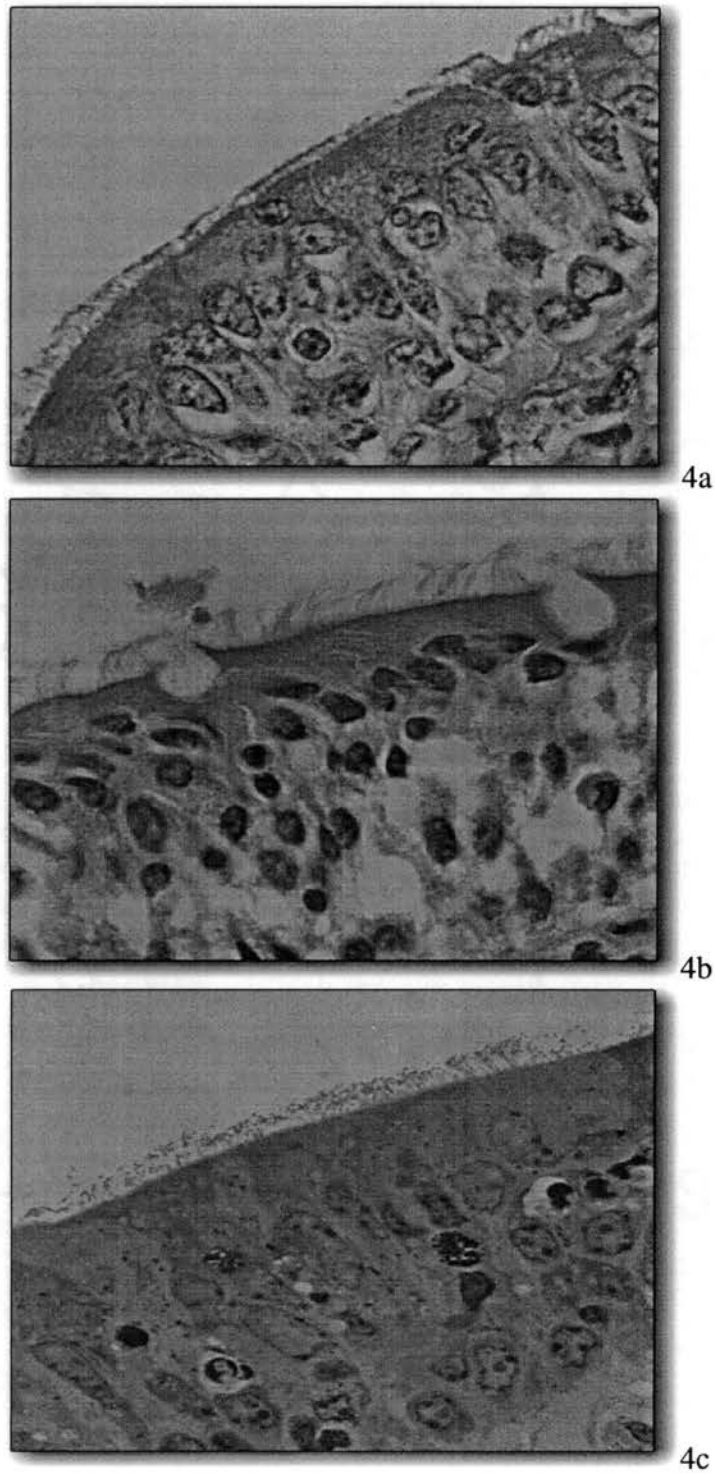
Figure 4. Photomicrographs of bovine nasopharyngeal tissue: a) control tissue incubated for 3 hours without bacteria, showing a healthy intact ciliated mucosal surface, b) tissue after 3 hours incubation with *Pasteurella haemolytica* A1 showing an intact ciliated epithelium with productive goblet cells, and c) tissue after 18 hours incubation with *P. haemolytica* A1 showing signs of cell damage with apototic nuclei, necrotic cells, and cilia loss (250 X).
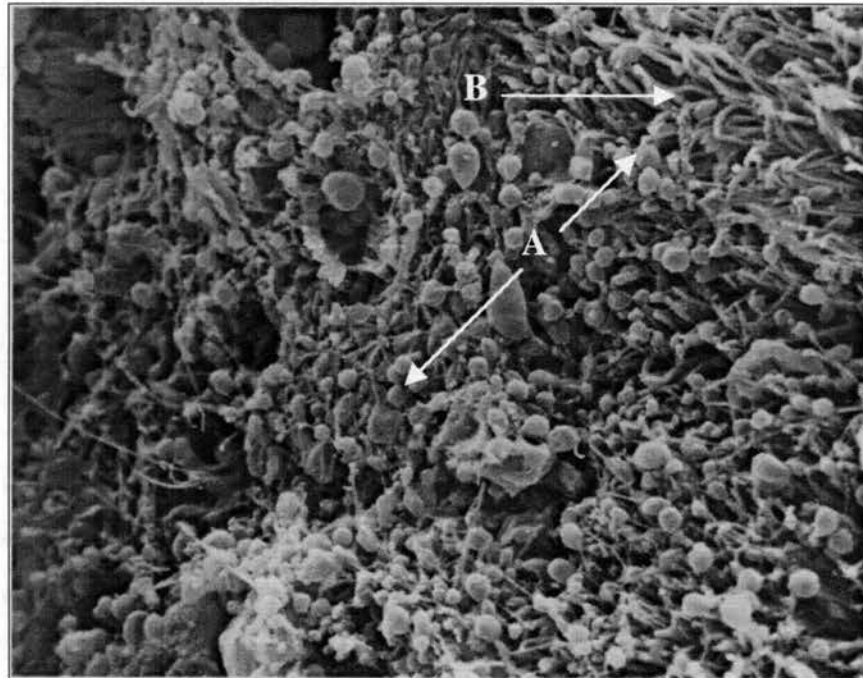
Figure 5. Scanning electron micrograph of bovine nasopharyngeal epithelium after 3 hours incubation with *Pasteurella haemolytica* A1. Note the presence of bacteria (A) adhered to cilia (B) on the surface of the tissue (2500 X).
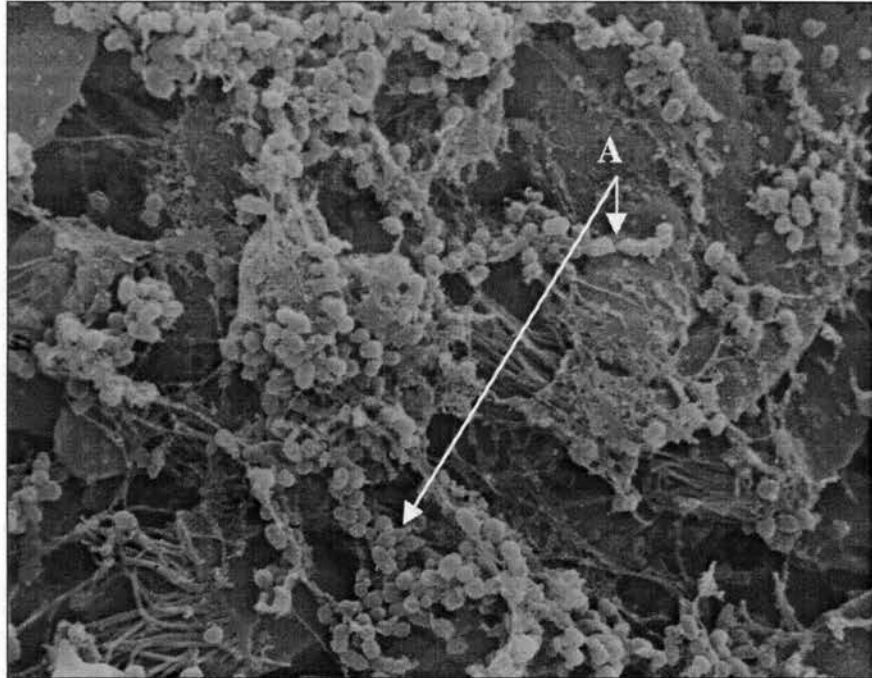
Figure 6. Scanning electron micrograph of bovine nasopharyngeal epithelium after 3 hours incubation with *Pasteurella haemolytica* A1. Note the presence of bacteria (A) aggregating along the lines of ciliation (2500 X).
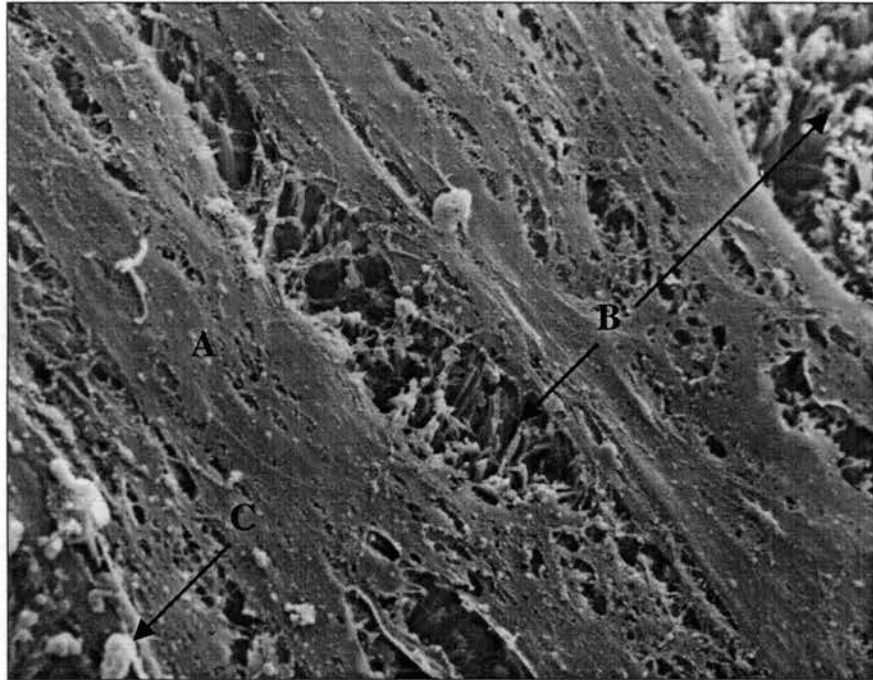
Figure 7. Scanning electron micrograph of bovine nasopharyngeal epithelium after 3 hours incubation with *Pasteurella haemolytica* A1. Note the layer of mucus (A) overlying cilia (B), with very few bacteria (C) present (2500 X).
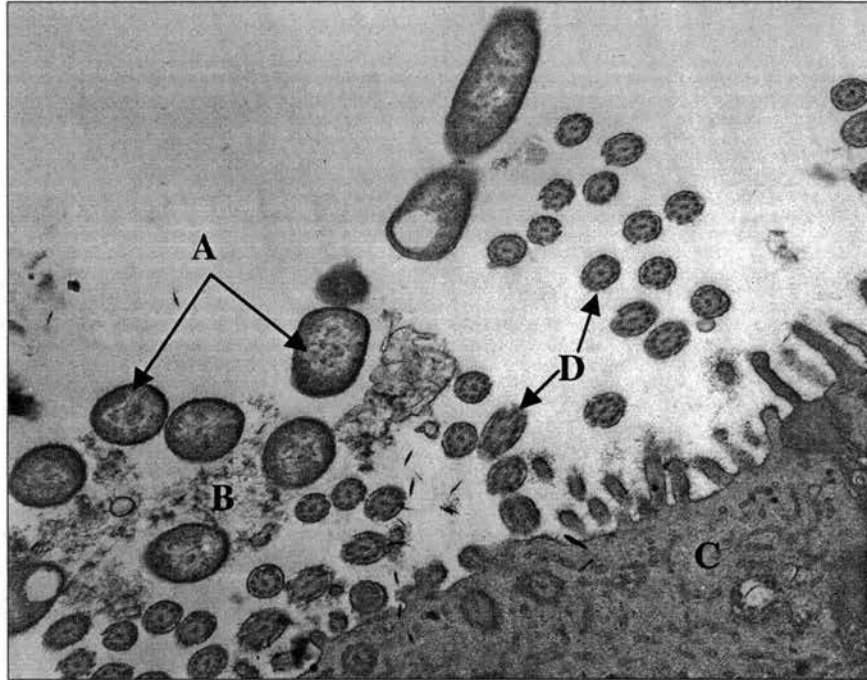
Figure 8. Transmission electron micrograph of bovine nasopharyngeal epithelium after 3 hours incubation with *P. haemolytica*. Note the presence of bacteria (A) in the mucus layer (B) above the ciliated epithelial surface (C) showing a number of cilia in cross-section (D) (16500 X).
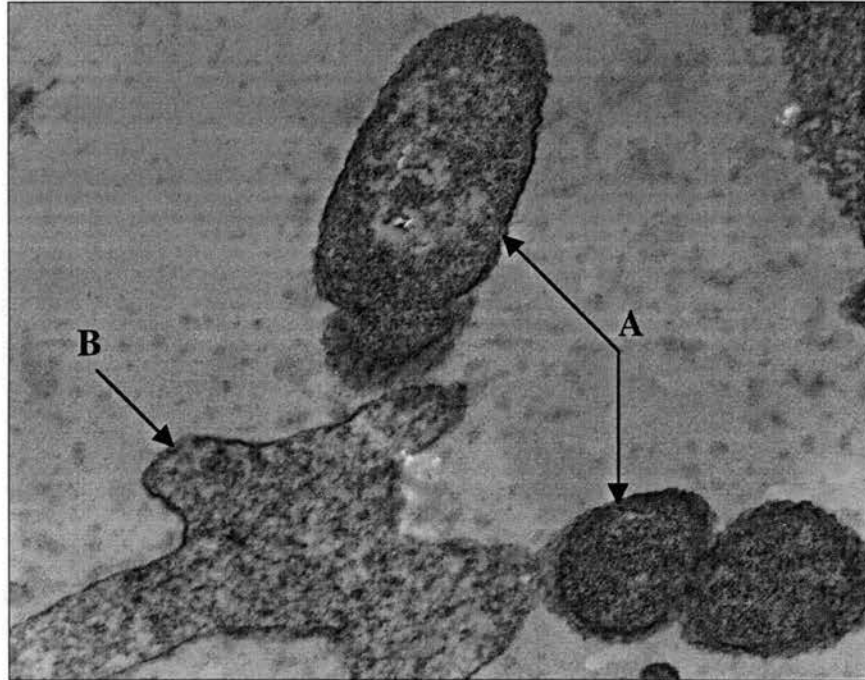
Figure 9. Transmission electron micrograph of *Pasteurella haemolytica* A1 bacteria (A) adhered to mucus (B) above the ciliated surface of bovine nasopharygeal epithelium (45000 X).
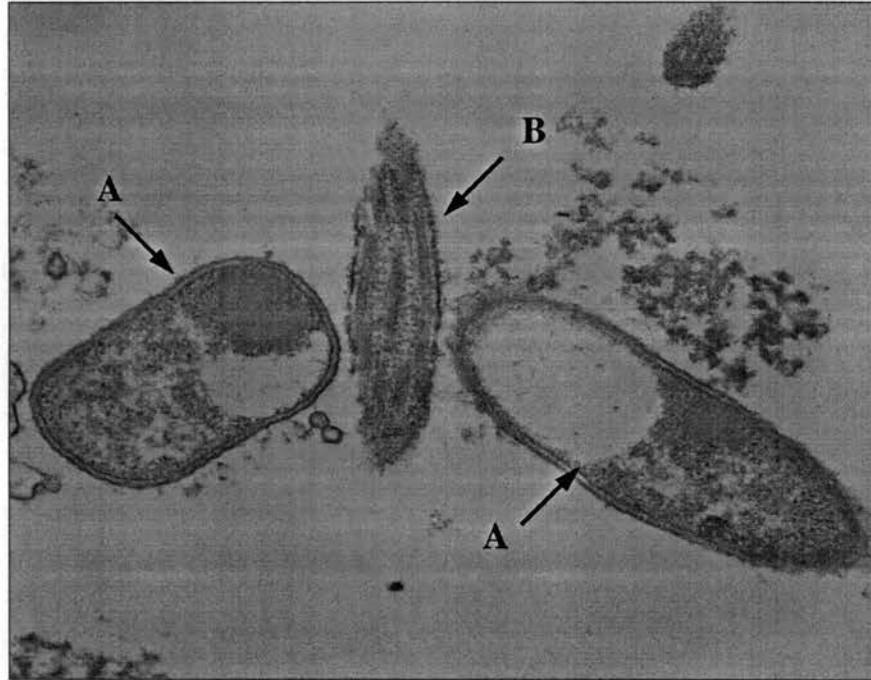
Figure 10. Transmission electron micrograph of two *Pasteurella haemolytica* bacteria (A) adhered to a cilium (B) after 3 hours incubation on bovine nasopharyngeal epithelium (45000 X).
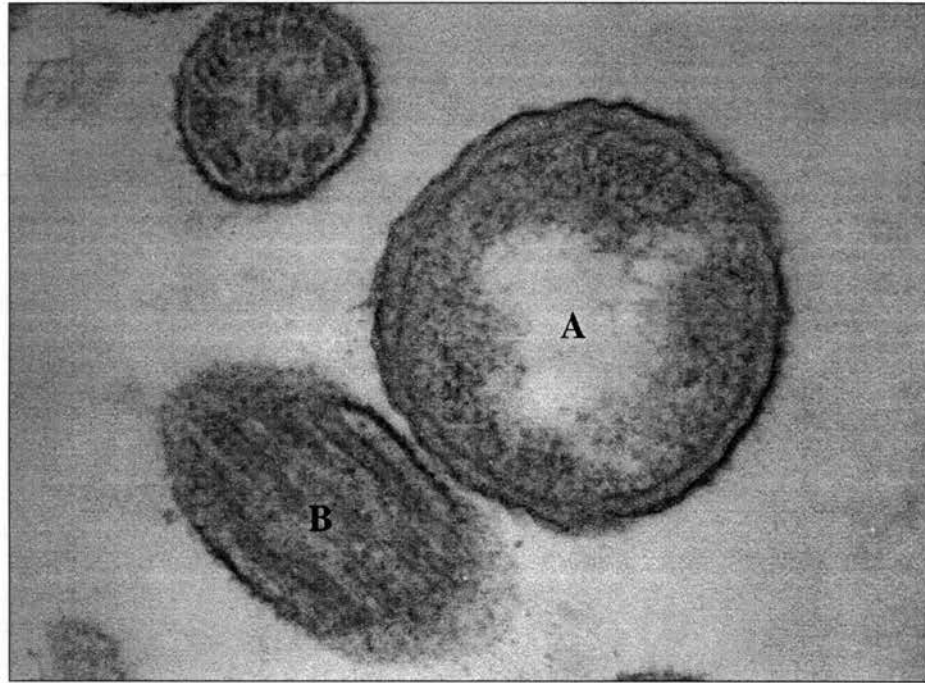
Figure 11. Transmission electron micrograph of a cross-section of *Pasteurella haemolytica* A1 (A) adhered to a cilium (B) after 3 hours incubation on bovine nasopharyngeal tissue (105000 X).
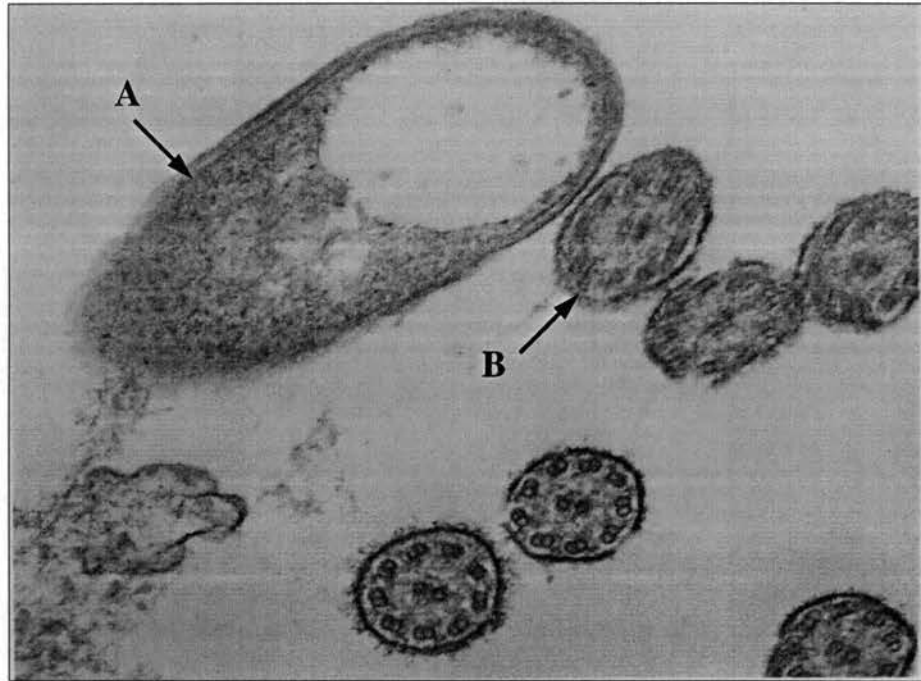
Figure 12. Transmission electron micrograph of a *Pasteurella haemolytica* bacterium (A) adhered to a cilium (B) after 3 hours incubation on bovine nasopharyngeal epithelium (60000 X).
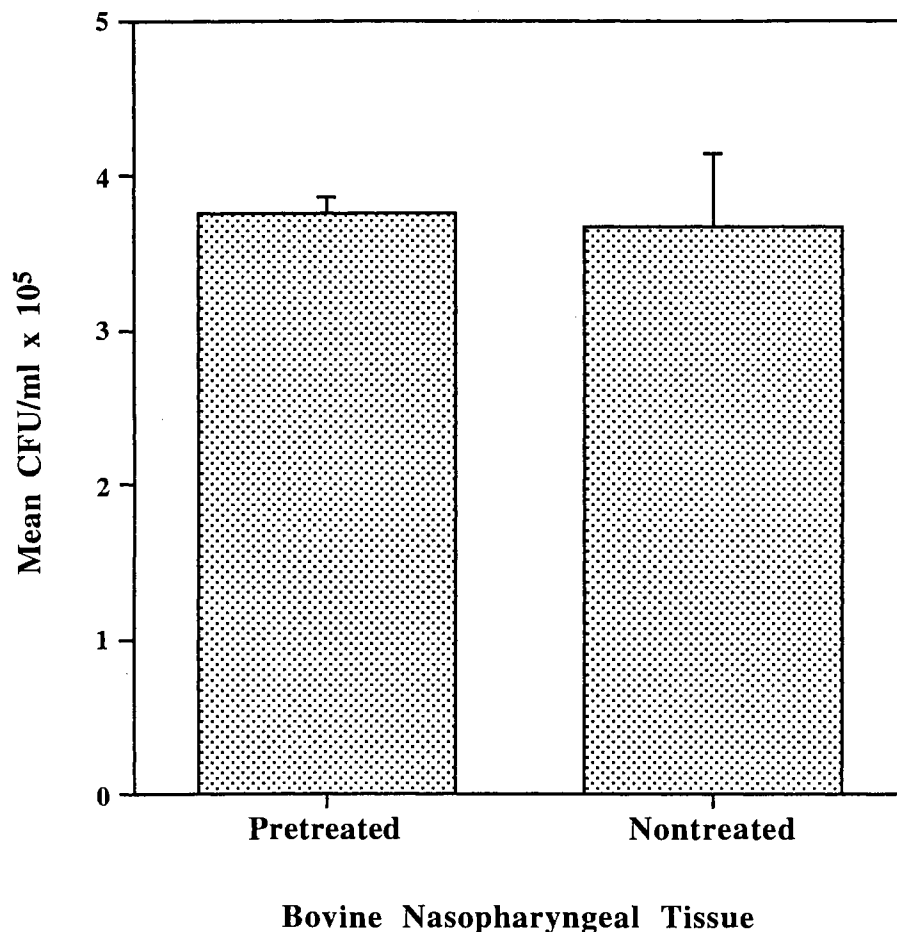
Figure 13. Effect of antibiotic pretreatment of bovine nasopharyngeal tissue on viability of *P. haemolytica*. Gentamicin and ampicillin-pretreated and nontreated nasopharyngeal tissue were inoculated with 50 μl undiluted inoculum and incubated at 37C for 3 hours. Tissue, inoculum, and PBS wash from each well were removed to a scintillation vial, vortexed, and radioactivity (Cpm) and bacterial concentration (CFU/ml) of the supernatant measured. Data represent the means ± SD (error bars) of one experiment with three replicates of each treatment group per experiment. No significant differences at P<0.01.
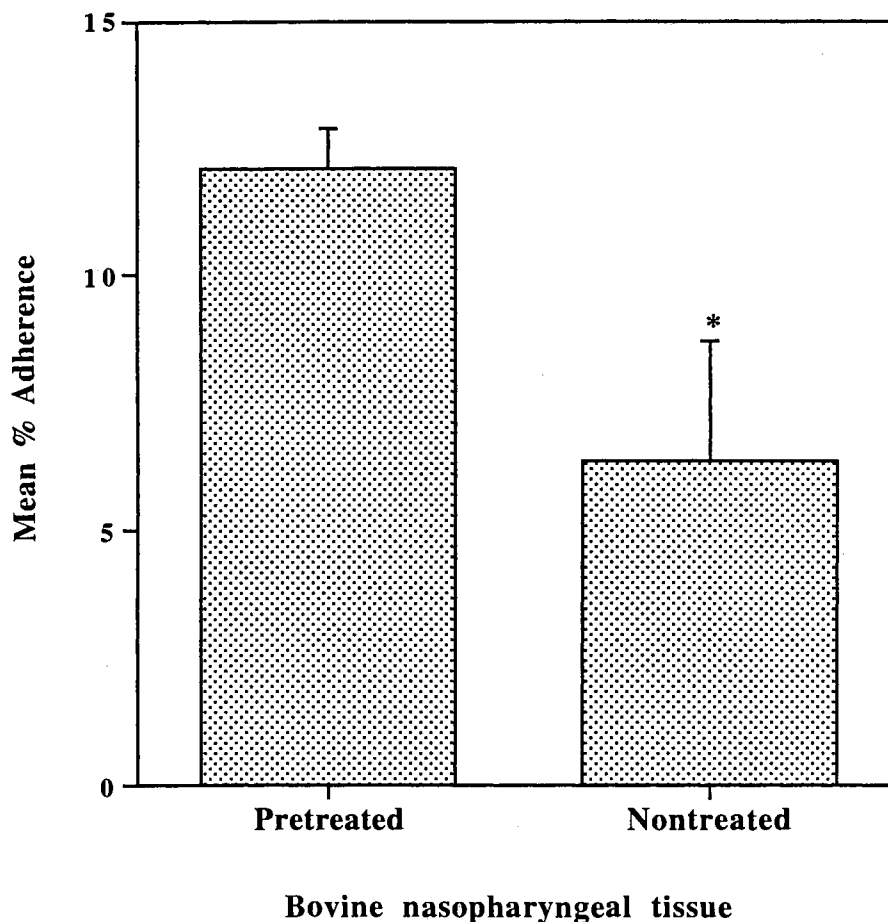
Figure 14. Effect of antibiotic pretreatment of bovine nasopharyngeal tissue on adherence of *P. haemolytica*. Adherence of PhA1 to gentamicin and ampicillin-pretreated and nontreated nasopharyngeal tissue were compared by inoculating each tissue with 50 μl PhA1 inoculum and incubating for 3 hours at 37C. Tissue was washed, removed to scintillation vials, digested, and radioactivity (Cpm) measured. Data represent the means ± SD (error bars) of one experiment with three replicates of each treatment group per experiment. * Significantly (P<0.01) less adherence to nontreated nasopharyngeal tissue.
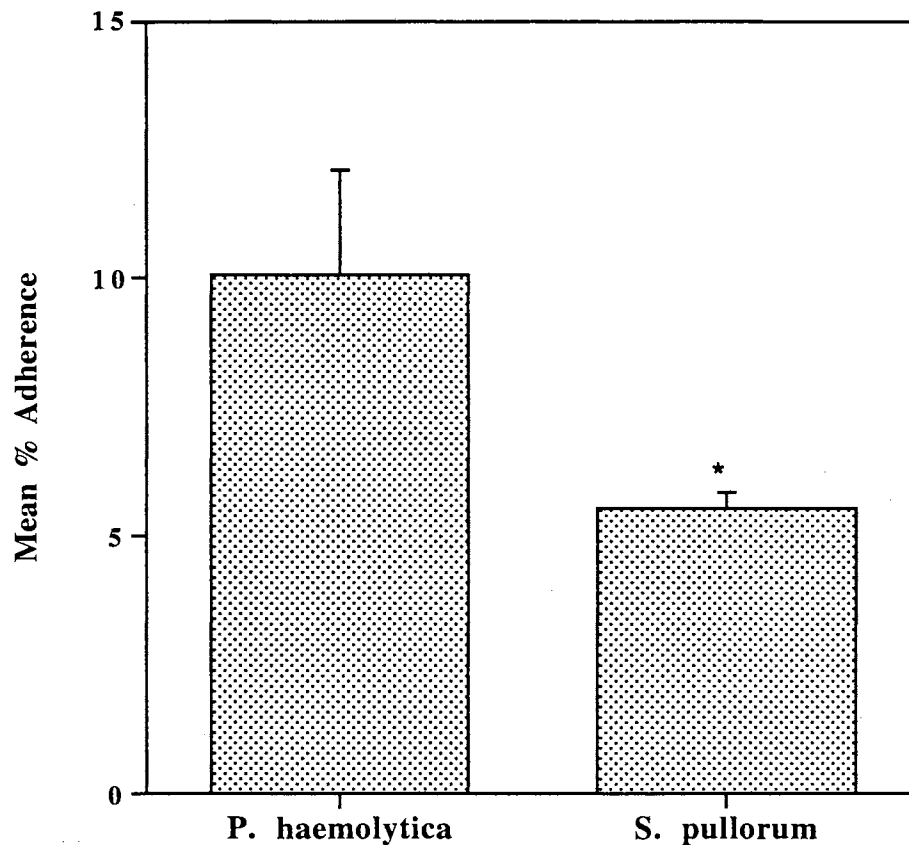
Figure 15. Comparison of adherence to bovine nasopharyngeal tissue of *Pasteurella haemolytica* and *Salmonella pullorum*. Nasopharyngeal tissue was incubated at 37C with 1 x 10^{7} CFU/ml of both organisms for 3 hours. Tissue was washed and radioactivity (Cpm) measured. Data represent the means ± SD (error bars) of one experiment with four replicates of each treatment group. * Significantly (P<0.05) less adherence of *S. pullorum* than *P. haemolytica* to nasopharyngeal tissue.
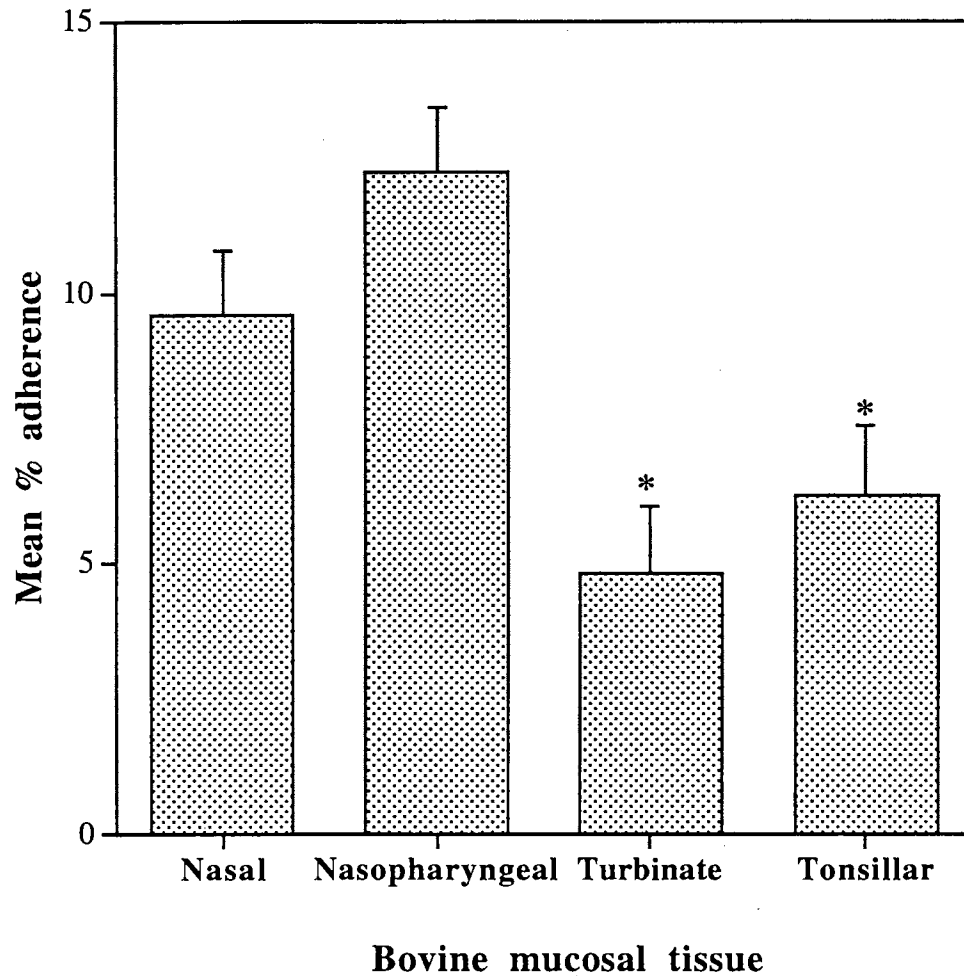
Figure 16. Effect of bovine upper respiratory tract tissue type on *P. haemolytica* A1 adherence. Nasal, nasopharyngeal, turbinate, and palatine tonsillar tissue were incubated at 37C with 1 x $10^7$ CFU/ml PhA1 for 3 hours. Tissue was washed and radioactivity (Cpm) measured. Data represent least square ($\pm$ SE) means from two experiments each with n = 5. *Significantly less than adherences to nasal and nasopharyngeal tissues.

CHAPTER VI

ADHERENCE OF *PASTEURELLA HAEMOLYTICA* TO BOVINE

NASOPHARYNGEAL TISSUE: EFFECTS OF CAPSULE, SEROVAR, AND BOVINE

HERPES VIRUS - 1

Introduction

*Pasteurella haemolytica* biotype A serovar 1 (PhA1) is the most frequently

isolated bacterium from both the lungs and nasal cavities of cattle suffering from acute

bovine respiratory disease (BRD) (Frank and Smith, 1983; Frank, 1979; Frank, 1984;

Allan *et al*, 1985). Research over the last 25 years has provided much insight into the

etiology and epidemiology of this disease (Frank, 1988) as well as the pathogenesis of

pulmonary infection (Slocombe *et al*, 1985; Clinkenbeard *et al*, 1994), but mechanisms

involved in initial bacterial colonization of the respiratory tract remain relatively

unexplored.

Frank has hypothesized that PhA1 exists as a commensal in very low numbers in

the nasopharynx and tonsils of healthy cattle (Frank, 1986; Frank and Smith, 1983; Frank

and Briggs, 1992) and that insults to the host defense system, such as respiratory viral

infection and/or stress, result in rapid multiplication of bacteria on the upper respiratory

tract (URT) epithelium (Frank and Smith, 1983; Frank, 1986; Frank *et al*, 1987; Jones,

1987). This dramatic increase in PhA1 numbers serves as a source of aerosolized

bacteria that enter the lung with inspired air, resulting in pulmonary infection.

Adherence is recognized to play an important role in colonization of mucosal surfaces by most bacteria (Beachey, 1981). Information relating specifically to colonization of bovine URT epithelium by *P. haemolytica*, however, is lacking due to the absence of suitable experimental models that could be used to study adherence. The recent development of *in vitro* and *ex vivo* adherence assays by the author has provided models for detailed investigation of interactions between *P. haemolytica* and URT epithelium. Using an *in vitro* fluorometric assay (Clarke and Morton, 2000), the author determined that adherence of PhA1 was significantly greater than that of *Pasteurella haemolytica* biotype A serovar 2 (PhA2), the serovar isolated most commonly from the nasopharynx of healthy cattle (Frank and Smith, 1983; Purdy *et al*, 1986). Greater adherence of PhA1, which is the serovar isolated most commonly from pneumonic animals, suggests that this serovar employs mechanisms of adherence that are not available to PhA2. Bacterial factors playing a role in *P. haemolytica* adherence have not yet been identified, but studies involving other respiratory bacteria have implicated fimbriae, outer membrane proteins, LPS, and capsule (St. Geme and Cutter, 1995; Barenkamp and Leininger, 1992; Bélanger *et al*, 1990; Favre-Bonte *et al*, 1995). Electron microscopy studies conducted by the author indicated that adherence of PhA1 to respiratory epithelium did not involve direct contact between bacterial and respiratory cytoplasmic membranes. This observation, together with the absence of identifiable fimbriae, suggests that adhesion may be facilitated by extracellular material, such as bacterial capsule.

Therefore, the objectives of this study were: to use an *ex vivo* model to confirm the results of an *in vitro* fluorometric assay (Clarke and Morton, 2000) demonstrating

differences in adherence between different serovars of *P. haemolytica*; and to explore the role of capsule in adherence of PhA1 and PhA2. The *ex vivo* model consists of functional bovine URT epithelium maintained in an *in vitro* environment and is more representative of respiratory tissue than the cell culture monolayers employed in the fluorometric *in vitro* assays. Additionally, the effect of bovine herpes virus - type 1 (IBR virus) infection on adherence of PhA1 to nasopharyngeal epithelium was investigated.

## Materials and Methods

### Radiolabeling of Bacteria

Bacteria used in experiments investigating the role of capsule were *Pasteurella haemolytica* A1 strain 668 (PhA1-668), a low passage strain isolated from the lung of a calf with pneumonic pasteurellosis (obtained from the Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK) and *Pasteurella haemolytica* A2 isolate #3 (PhA2-3), which was isolated from bovine nasal passages (provided by Dr. Glynn Frank, NADC, Ames, Iowa). Studies comparing adherence of different serovars employed PhA1-668, PhA2-3 and a variety of other *P. haemolytica* A1, A2, and A6 isolates (also provided by Dr. Glynn Frank).

Bacteria were radiolabeled as previously described. Briefly, bacteria were cultured, harvested in leucine-deficient Roswell Park Memorial Institute media (ICN Biomedicals, Inc.) plus 1 ug/ml L-glutamine (JRH Biosciences) (RPMI -), and grown to midlog phase (estimated from a previously constructed growth curve). Two μCi/ml of tritiated leucine ($[^3H]$-leucine) (ICN Pharmaceuticals, Inc.) was incubated at 37C with the

bacterial inoculum for 1 hour. Labeled bacteria were centrifuged at 8000 rpm (Sorvall RC2-B, rotor SM-24), washed in PBS and refrigerated overnight, after which the pellet was resuspended in PBS, washed, and diluted to 1 X $10^7$ CFU/ml. Radioactivity was measured in Atom-Light liquid scintillant (Packard Instrument Co., Inc.) using a liquid scintillation counter (Model LS 5000TD, Beckman Instruments Inc.).

Tissue Collection and Experimental Procedure

Tissue collection and assay were conducted as described previously. Briefly, nasopharyngeal tissue collected from freshly slaughtered steers after resecting and exposing the nasopharynx was checked for an undamaged surface, beating cilia, and mucus production. Tissue was placed in media containing antibiotics (RPMI with L-glutamine, 20 µg/ml gentamicin (Sigma Chemical Co.) and 20 µg/ml sodium ampicillin {Marsam Pharmaceutical, Inc.}), incubated for 1 hour at 37C, washed, and then reincubated in antibiotic-free media for a further hour. Small, uniform tissue samples were prepared using a 6 mm diameter biopsy punch, incubated for another hour, and then embedded in 15 ul molten agarose (Sigma Chemical Co.) contained in wells of a 96-well tissue culture plate (Corning Glass Works). The agar was allowed to cool and solidify, thus preventing bacterial adherence to the underside and sides of the tissue samples. Fifty µl of prepared radiolabeled bacterial inoculum was pipeted into each well and incubated for 3 hours at 37C. Nonadherent bacteria were removed by washing the tissue 5x with sterile PBS and tissue samples were then removed from the wells, residual agar scraped from the underside and sides of the tissue, and each sample digested overnight in Soluene-350 (Packard Instrument Co.). Radioactivity of digested samples was measured

in Atom-Light using a liquid scintillation counter. Radiolabeled inocula were also subjected to direct scintillation counting to obtain total radioactivity of bacteria added to the tissue samples.

Effect of Capsule on Adherence

Radiolabeled PhA1-668 and PhA2-3 were decapsulated by incubating for 1 hour in phosphate buffered saline (PBS, pH 7.3 - 7.4) at 41C and 120 oscillations/minute in an incubator shaker (Innova 4000 Incubator Shaker, New Brunswick Scientific Co., Inc) (Gentry *et al*, 1982). The success of capsule removal was confirmed by subjecting capsulated and decapsulated PhA1-668 to light microscopy using Maneval stain (Figs. 1a and 1b) and to TEM using negative-staining (Figs. 2a and 2b). Inocula containing capsulated or decapsulated bacteria were incubated on nasopharyngeal tissue at 37C for 3 hours and tissue was washed 5x with PBS, removed, digested, and radioactivity was measured. The experiments was repeated three times (n=5) and data comparing adherences of the capsulated and decapsulated isolates were analyzed using a 2 x 2 factorial arrangement of treatments with repeated experiments represented as blocks. Significance was declared at the P<0.05 level.

In order to test whether adherence of capsulated bacteria to nasopharyngeal tissue could be blocked, PhA1 capsular extract was incubated at 37C on bovine nasopharyngeal tissue for 1 hour, excess extract was aspirated from the tissue surface, and the tissue gently washed once with PBS. Capsulated PhA1-668 was then added and incubated on the tissue at 37C for 3 hours (n=5 for each of 3 experiments). Capsular extract was obtained by PBS extraction; PhA1-668 was grown in BHI broth for 6 hours in an

incubator shaker at 37C, centrifuged, and the pellet resuspended in 100 ml prewarmed (41C) sterile PBS. The culture was then incubated at 41C for 1 hour in the incubator shaker, centrifuged, and the supernatant filter-sterilized and concentrated using dialysis in Aquacide II (Calbiochem, Behring Diagnostics, Hoechst). The osmolality of the extract was adjusted to an approximate bovine physiological level, i.e. 272 mosm/L, and used undiluted on the tissue surface. Phosphate buffered saline was used as a control.

To test whether adherence by capsule was specific, undiluted bovine PhA1 capsular extract antibody (supplied by Dr. A. Confer's laboratory, Department of Anatomy, Pathology and Pharmacology, Oklahoma State University) was incubated at 37C for 1 hour with capsulated PhA1-668 (1:1). Fifty ul was added to each nasopharyngeal tissue piece (n = 5) and incubated for 3 hours at 37C, after which, the tissue was washed, digested and radioactivity measured. Capsulated PhA1-668 was also incubated with fetal bovine serum (FBS) as a control.

Effect of Serovar on Adherence

Two different strains of each of three serovars (PhA1, PhA2, and PhA6) were radiolabeled and used in the adherence assay. These bovine isolates were PhA1-668 and PhA6-D198 of pneumonic lung origin, and PhA1-1, PhA2-8, PhA2-3, and PhA6-4 of nasal origin. All nasal isolates were obtained from calves that had been recently shipped to a feedlot in New Mexico. PhA6-4 was isolated from a calf suffering from acute BRD. The experiment was repeated twice, with three replicates per isolate for each experiment. Data were analyzed using analyses of variance with repeated experiments represented as

blocks, and isolate differences were analyzed by Scheffe's Test. Significance was declared at the P<0.05 level.

Effect of Preincubation of Nasopharyngeal Tissue with IBR Virus on PhA1 Adherence

Prepared nasopharyngeal tissue was incubated at 37C with 50 ul of either 1:100, 1:1000, or 1:10,000 dilutions of IBR virus ($10^6$ $TCID_{50}$/ml; obtained from Dr. J. d'Offay, Department of Infectious Diseases and Physiology, College of Veterinary Medicine, Oklahoma State University) or with no virus (controls), in Earle's Minimum Essential Media (MEM) for 24 hours. Viral-infected media was then aspirated and tissue washed once with PBS. One replicate of each viral-treated tissue piece was placed in Bouin's fixative for histopathology. Tissue replicates were reincubated at 37C with 50 ul of capsulated PhA1-668. After 3 hours and 18 hours (n=3 at each time for each viral dilution), replicates of tissue were washed 5x with PBS, removed, digested and radioactivity measured. The remaining replicates were either fixed in Bouin's fixative for histopathology or in glutaraldehyde for scanning electron microscopy (SEM). Control tissue without virus, and without virus and bacteria were also fixed for histopathology. Data comparing the adherence of PhA1-668 on the three different viral-pretreated tissues and control tissue at 3 and 18 hours were analyzed and significance was declared at the P<0.05 level. Histopathology sections were hematoxylin-eosin stained and viewed using light microscopy for viral damage to the epithelial surface, and for bacterial adherence. Tissue for SEM was fixed in 2% cacodylate-buffered glutaraldehyde overnight at 4C, followed by 3 x 20 minute 0.1M cacodylate buffer washes. The tissue was post-fixed in 1% cacodylate-buffered osmium tetroxide for 2 hours at 4C, followed by 3 buffer washes

and dehydration in a graded series of ethanol. Tissue was critical point dried, coated with gold/palladium, and viewed using SEM (JEOL JSM 35U) operated at 25kV.

## Results

### Effect of Capsule on Adherence

After 3 hours incubation at 37C on bovine nasopharyngeal tissue, followed by washing 5x with PBS to remove nonadherent bacteria, tissue digestion, and radioactivity assay, mean % adherence was calculated for both capsulated and decapsulated PhA1 and PhA2 isolates. Mean % adherence of capsulated PhA1-668 was significantly higher (P<0.05) than that of decapsulated PhA1-668 and both capsulated and noncapsulated PhA2-3. The mean % adherence for all 3 experiments for capsulated PhA1-668 was approximately 8 - 10%, compared to approximately 4 - 5% for decapsulated PhA1 and capsulated and decapsulated PhA2 (Fig. 3).

After preincubation of bovine nasopharyngeal tissue with PhA1 capsular extract, followed by incubation with capsulated PhA1-668, tissue washing, digestion, and assay of radioactivity, mean % adherence of PhA1-668 decreased significantly (P<0.05), indicating that capsule has a blocking effect. This would support the result indicating that capsulated PhA1-668 adheres to nasopharyngeal tissue. The blocking effect also indicated that capsulated PhA1-668 probably does not adhere to other capsule, or at least not tightly enough to prevent removal with washing of tissue. The PBS control used with PhA1-668 did not significantly affect its adherence (P<0.05).

After preincubation of capsulated PhA1-668 with bovine PhA1 capsular extract antibody, mean % adherence on nasopharyngeal tissue decreased significantly (P<0.05), indicating that adherence due to capsule may be specific. The FBS control did not significantly affect adherence (P<0.05) (Fig. 4).


## Effect of Serovar on Adherence

Mean % adherence to bovine nasopharyngeal tissue of both PhA1-668 and PhA6-D198 was significantly greater (P<0.05) than that of PhA1-1, PhA2-3, PhA2-8, and PhA6-4. The difference between PhA1-668 and PhA2-3 % adherence was consistent with that of previous capsule experiments. There were no significant differences (P<0.05) between mean % adherence of all nasal isolates. Thus, the lung isolates, i.e., PhA1-668 and PhA6-D198, adhered better than the nasal isolates. (Fig. 5).


## Effect of Preincubation of Nasopharyngeal Tissue with IBR Virus on PhA1 Adherence

Preincubation of bovine nasopharyngeal tissue with varying dilutions of IBR virus for 24 hours appeared to cause some damage to nasopharyngeal tissue i.e. localized loss of cilia and epithelial cell surface damage at all virus dilutions, as viewed in the histopathology hematoxylin-eosin stained preparations and in SEM. This damage remained similar even after 3 hours incubation with bacteria, but was more evident after 18 hours bacterial incubation, i.e. complete loss of cilia with damage and loss of epithelium, exposing large areas of underlying basement membrane, as viewed in histopathologic and SEM preparations (Figs 6 and 7, respectively).

Mean % adherence of PhA1-668 after 3 hours incubation was not significantly different on all viral-pretreated bovine nasopharyngeal tissue, and was very low compared to adherence of bacteria on tissue not pretreated with virus. There was a significant increase in adherence (P<0.05) on all viral-pretreated tissue by 18 hours, but again, this was low when compared to adherence to tissue not pretreated with virus (Fig 8).

## Discussion

Studies employing a fluorometric *in vitro* assay (Clarke and Morton, 2000) and an *ex vivo* radioactivity assay have demonstrated that PhA1 adheres to bovine URT cells, particularly nasopharyngeal epithelium. These observations are significant in that adherence is usually a prerequisite for colonization of a mucosal surface, allowing bacteria to multiply to high enough numbers for the infection to spread to other tissues (Beachey, 1981). In the case of *P. haemolytica* infection, initial colonization of the URT is hypothesized to precede development of fibrinopurulent bronchopneumonia.

Very little is known about URT adherence of *P. haemolytica* and neither bacterial adhesins nor host cell receptors have been definitively identified. One of the potential adhesins of *P. haemolytica* is capsule, although there is evidence that it is a poor immunogen, as demonstrated by an experiment in which vaccination of calves with *P. haemolytica* capsular polysaccharide did not confer protective immunity (Conlon and Shewen, 1993). In experiments conducted to test the effect of capsule on adherence of strains of PhA1 and PhA2 to bovine nasopharyngeal tissue, capsulated PhA1-668 adhered

significantly more than decapsulated PhA1-668 and capsulated and decapsulated PhA2-3. Subsequent experiments indicating that PhA1 capsule extract and capsular antibody could block adherence of capsulated PhA1-668 confirmed the involvement of capsule in specific adherence of *P. haemolytica* to nasopharyngeal tissue. It has been postulated that *Haemophilus influenzae* type b, which is encapsulated, adheres loosely to the epithelial surface during log-phase growth, possibly via long pili that emerge through the capsule surface. As the thickness of the capsule decreases during stationary phase, adherence becomes stronger and more specific via shorter fibrils which are then exposed (St. Geme and Cutter, 1996). A similar mechanism of adherence could be employed by *P. haemolytica*, although only putative pili have been observed for this organism to date (Morck *et al*, 1987 and 1988; Gilmour *et al*, 1985; Potter *et al*, 1988), and none were observed in the present experiments.

The presence or absence of capsule was confirmed using Maneval's stain as well as negative-staining and TEM prior to inoculation of nasopharyngeal tissues. However, when adhered bacteria were examined by TEM after they had been incubated with nasopharyngeal tissue *ex vivo* (see previous study), very little capsule was observed around capsulated bacteria. This is not altogether surprising as there is evidence that bacteria cultured *in vitro* have a less distinctive polysaccharide capsule than is found after replication *in vivo* (Brogden and Clarke, 1997; Jacques *et al*, 1994) and St. Geme and Cutter (1996) have suggested that encapsulation may be down-modulated during the early infectious process. Another explanation for the apparent lack of capsule may be related to the TEM processing itself. Bacterial glycocalyx is an exopolysaccharide composed of >95% water (Bayer *et al*, 1995), which may be removed by alcohol

dehydration during processing, leaving the glycocalyx fibers collapsed and condensed down onto the cell surface and almost invisible unless they are stabilized with antibody and stained with an appropriate stain such as ruthenium red (Morck *et al*, 1988; Jacques *et al*, 1988; Wagner *et al*, 1982). Therefore, the absence of a thick visible capsule on TEM does not preclude its existence or the possibility that capsule plays a role in adherence.

It has been well-established that PhA1 and PhA2 possess capsules that differ in their composition, and therefore could differ in their ability to adhere, as well as their ability to protect the organism against the host's immune system. The capsule of PhA1 is a mannopyranosyluronic acid derivative, whilst that of PhA2 is an acetylneuraminic acid derivative (Adlam *et al*, 1984; Adlam *et al*, 1987). The differences in the type of capsule may indicate why capsulated PhA1-668 adhered significantly more than capsulated PhA2-3 and could explain differences in virulence between serovars. The predominance of PhA1 in the etiology of BRD versus the existence of PhA2 as a commensal in the nasal passages of healthy cattle (Frank and Smith, 1983; Frank, 1979 and 1984; Purdy *et al*, 1986) is an intriguing phenomenon of the disease. It appears that PhA1 possesses certain virulence factors that allow it not only to cause the extensive lung damage of BRD, but to gain an initial foothold in the URT of stressed or viral-infected calves. Likewise, the higher adherence of PhA6 correlates with the involvement of this serovar in pneumonic pasteurellosis. PhA6 strains have a similar protein profile to those of PhA1 (Morton *et al*, 1994) and are isolated more frequently from pneumonic lungs than other serovars, except PhA1. PhA6 serovars also have been shown to induce cross-protection in mice against PhA1 challenge (Donachie *et al*, 1984). Results indicating that PhA1 and

PhA6 strains originally isolated from the lungs of animals that had died from pneumonic pasteurellosis adhered to a greater degree than strains and serovars isolated from the URT of living animals adds further credence to the probability that differences in virulence between *P. haemolytica* serovars and strains is related to their ability to adhere and colonize the URT.

Previous studies by Frank and coworkers have demonstrated that PhA1 only undergoes rapid multiplication in the URT when animals are physically stressed and/or infected by respiratory viruses, such as IBR-1 virus (Frank and Smith, 1983; Frank, 1986; Frank *et al*, 1987; Jones, 1987). When nasopharyngeal tissue was exposed for 24 hours to various dilutions of IBR-1 before inoculation of PhA1, adherence of bacteria at 3 hours was significantly lower than that of bacteria incubated with tissue not exposed to virus. By 18 hours of incubation, bacterial adherence had increased, probably due to bacteria becoming trapped on the eroded epithelial surface, but was still significantly lower than that observed in the absence of virus exposure. These results suggest that IBR viral infection does not predispose animals to *P. haemolytica* infection by promoting adherence, unlike mucosal colonization by some other bacteria (Read *et al*, 1991), but that other effects on pathogenesis are involved. It is possible that viral damage to the epithelial cells in the present study decreased bacterial adherence by eliminating host cell receptors on cilia or the epithelial surface or by changing the structure of the mucus to which bacteria would normally adhere.

In conclusion, studies conducted using an *ex vivo* assay of adherence have confirmed that *P. haemolytica* adheres to bovine URT epithelium and have provided strong circumstantial evidence that the greater virulence of PhA1 strains isolated from

lungs of pneumonic animals may be related to their ability to adhere to and colonize the URT. Preliminary experiments exploring the involvement of bacterial factors in adherence have suggested an important role of capsule. It is expected that the availability of the *ex vivo* model employed in the present study will greatly facilitate future experiments designed to elucidate mechanisms of *P. haemolytica* adherence more definitively.
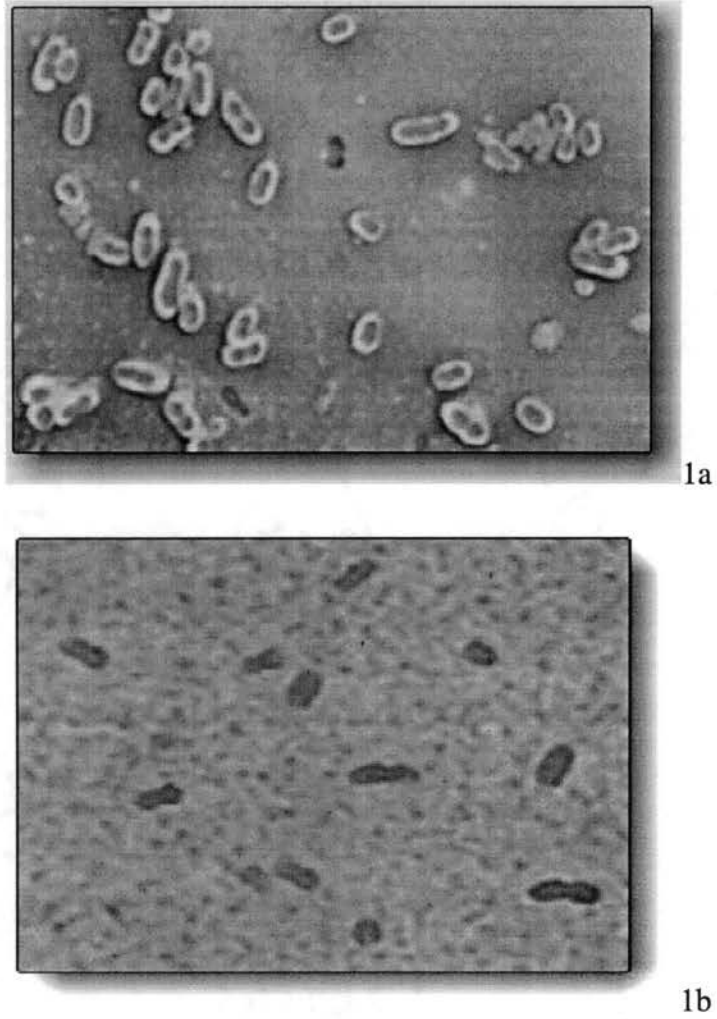
Figure 1. Photomicrographs of *P. haemolytica* A1 stained with Maneval stain. Note a) the presence of capsule prior to incubation with bovine nasopharyngeal tissue, and b) the absence of capsule after decapsulation by incubating capsulated bacteria in PBS at 41C for 1 hour (400 X).
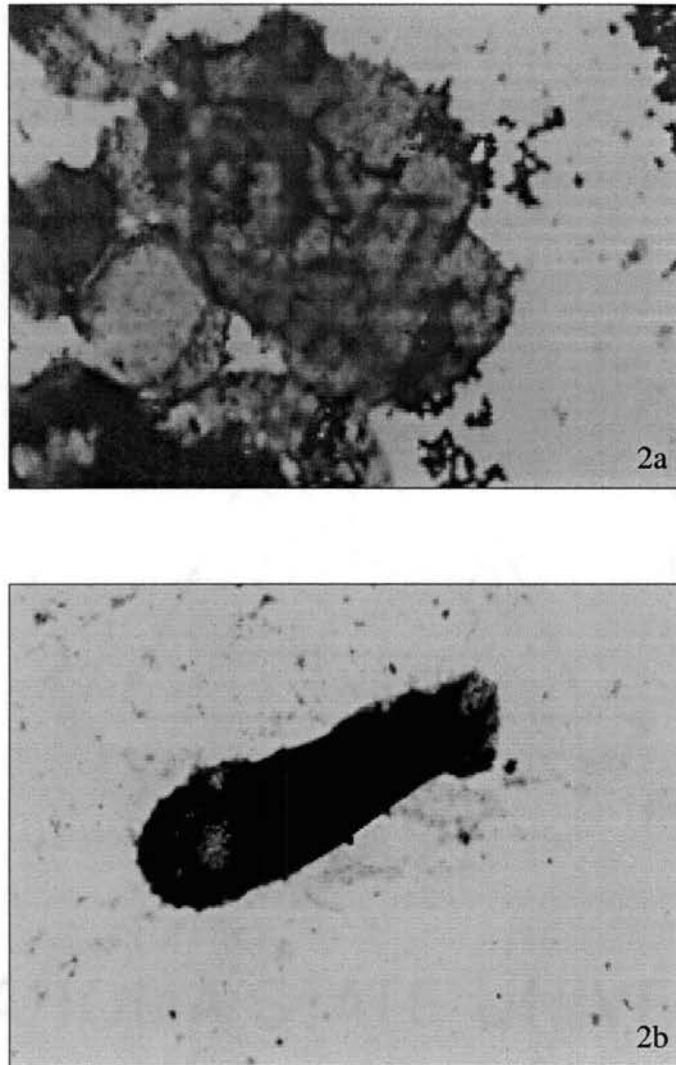
Figure 2. Transmission electron micrographs of negatively-stained *Pasteurella haemolytica* A1 showing a) capsulated bacterium and b) decapsulated bacterium, in inoculum prior to 3 hours incubation on bovine nasopharyngeal tissue (10000 X).
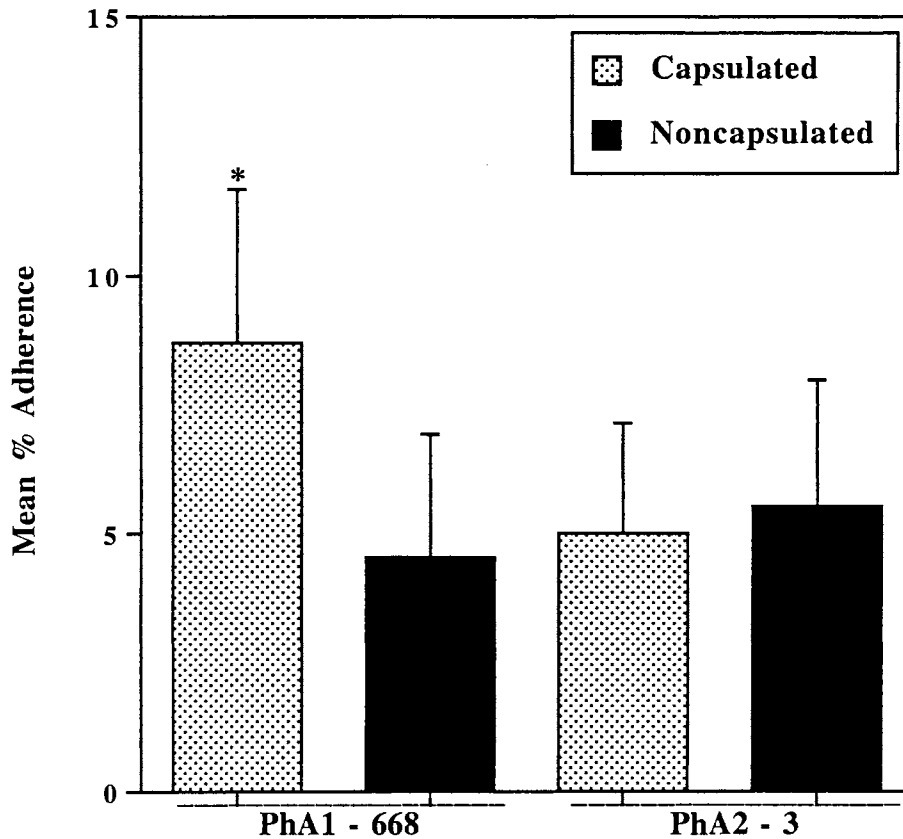
Figure 3. Effect of *P. haemolytica* A1-668 and *P. haemolytica* A2-3 capsule on adherence to bovine nasopharyngeal tissue. Log phase bacteria were radiolabeled, after which half of the inoculum for each isolate was incubated for 1 hour in PBS at 41C and 120 oscillations/minute to remove the capsule. The inocula of both capsulated and noncapsulated bacteria for each of the isolates were diluted to approximately 1 x $10^7$ CFU/ml, incubated on nasopharyngeal tissue at 37C for 3 hours, tissue washed 5x with PBS, removed, and digested, and radioactivity (Cpm) measured. Data represent the means ± SE (error bars) of three experiments with five replicates of each treatment group per experiment. *Significantly (P<0.05) greater than adherence of noncapsulated PhA1 and capsulated and noncapsulated PhA2.
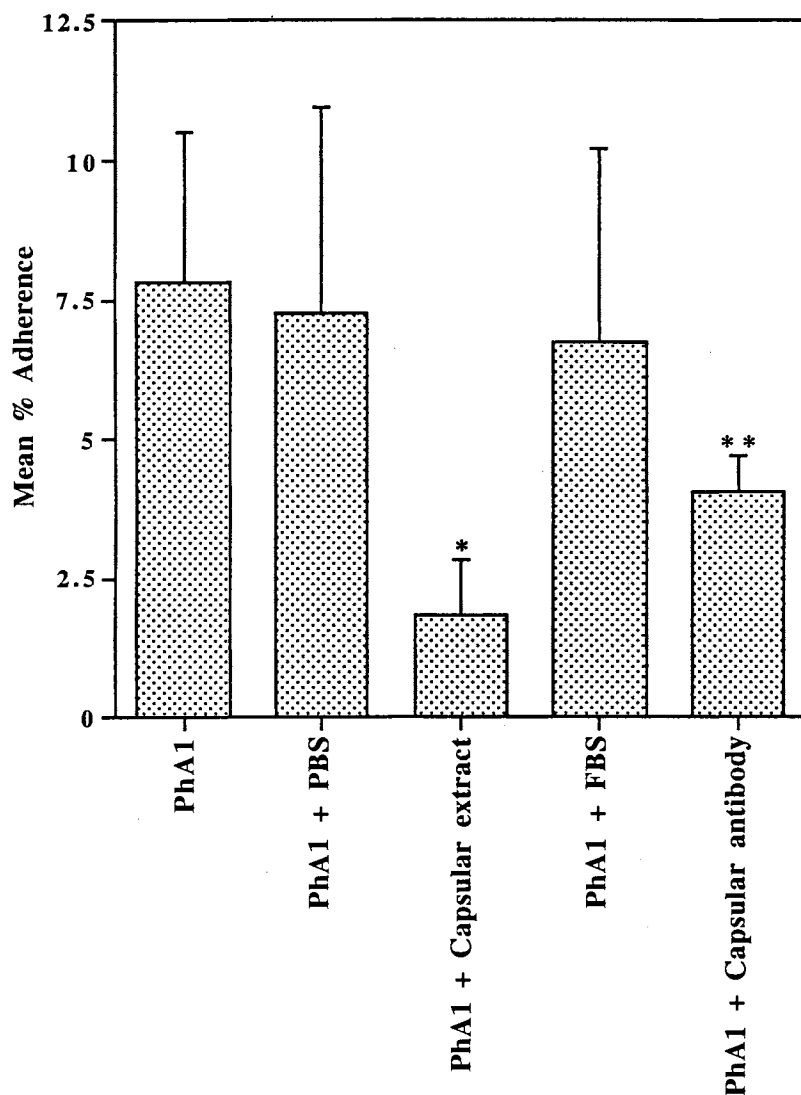
Figure 4. Effect on adherence of *P. haemolytica* A1-668 to bovine nasopharyngeal tissue by blocking with PhA1 capsular extract and bovine capsular extract antibody. After preincubation of nasopharyngeal tissue for 1 hour at 37C with capsular extract of PhA1-668, removal of excess extract, and washing of tissue, and after preincubation of radiolabeled PhA1-668 with PhA1 capsular extract antibody for 1 hour at 37C, radiolabeled capsulated PhA1-668 was incubated on tissue at 37C for 3 hours. Tissue was washed and digested, and radioactivity (Cpm) measured. Data represent the means ± SE (error bars) of three experiments with five replicates of each treatment group per experiment. * Significantly (P<0.05) less than adherence of PhA1 without capsular extract blocking (PBS only). ** Significantly (P<0.05) less than adherence of PhA1 without capsular extract antibody (FBS only).
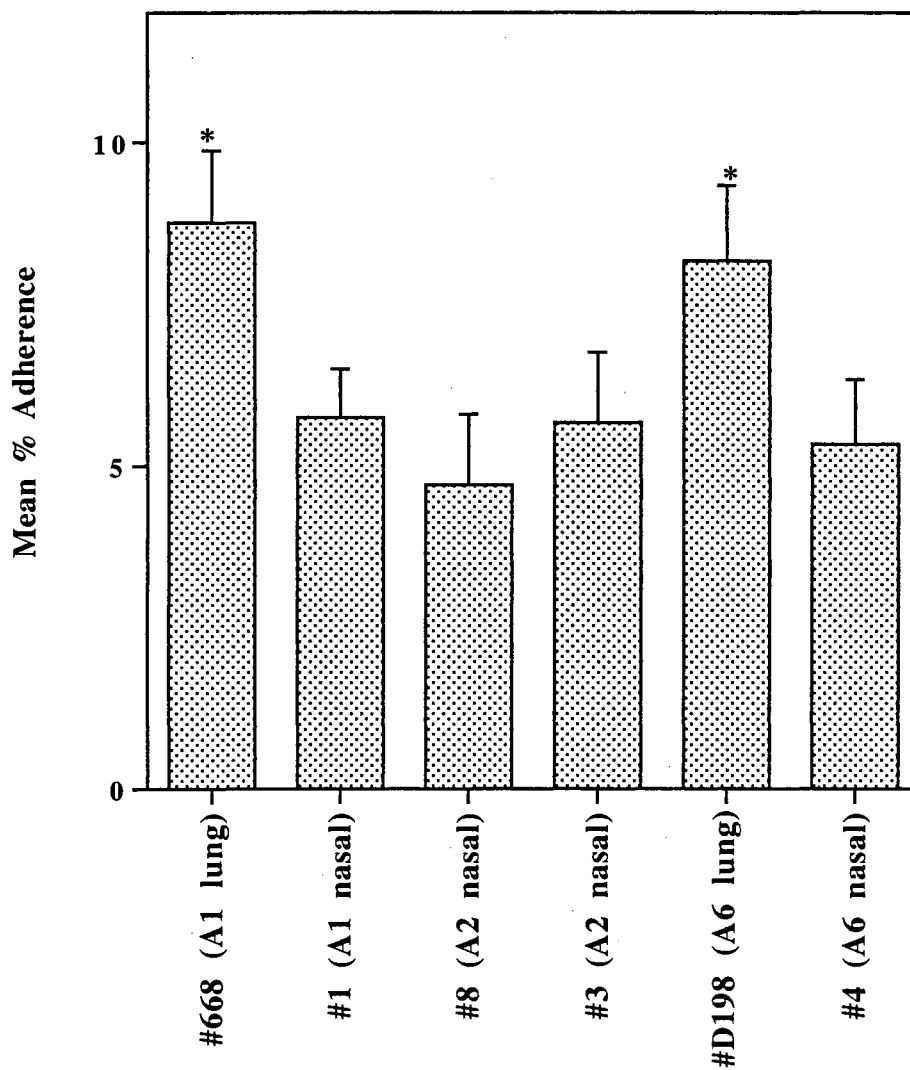
Figure 5. Adherence of two different strains each of three *P. haemolytica* serovars (PhA1, PhA2, and PhA6) to bovine nasopharyngeal tissue. Bacteria were radiolabeled, diluted to a concentration of 1 x $10^7$ CFU/ml, incubated on tissue at 37C for 3 hours, and tissue was washed and digested, and radioactivity of adherent bacteria (Cpm) measured. Data represent the means ± SE (error bars) of two experiments with three replicates of each isolate per experiment. *Significantly (P<0.05) greater than adherence of nasal isolates.
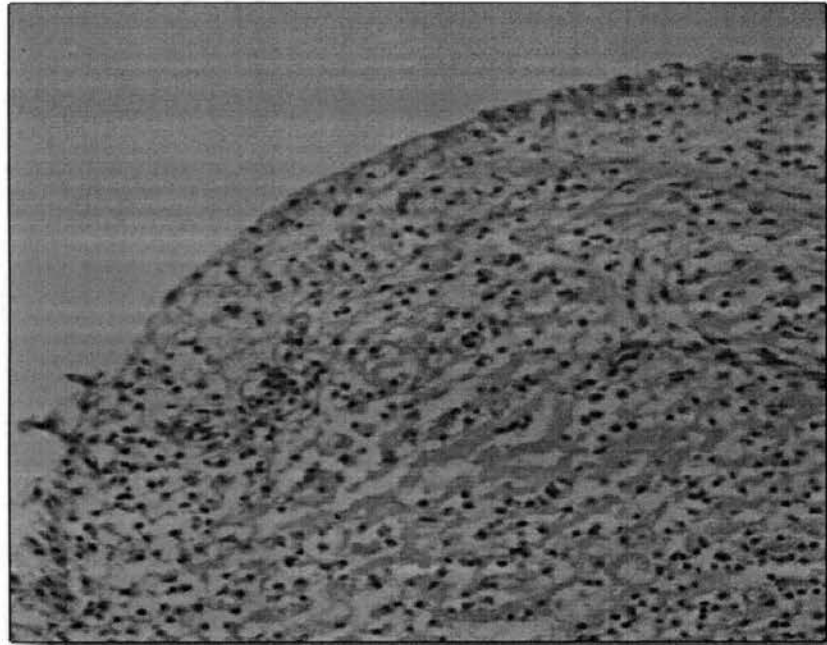
Figure 6. Photomicrograph of hematoxylin-eosin-stained bovine nasopharyngeal epithelium after 24 hours incubation with IBR-1 virus and a further 18 hours incubation with *P. haemolytica* A1. Note the damage to the epithelial layer with loss of epithelial cells and almost all cilia (50 X).
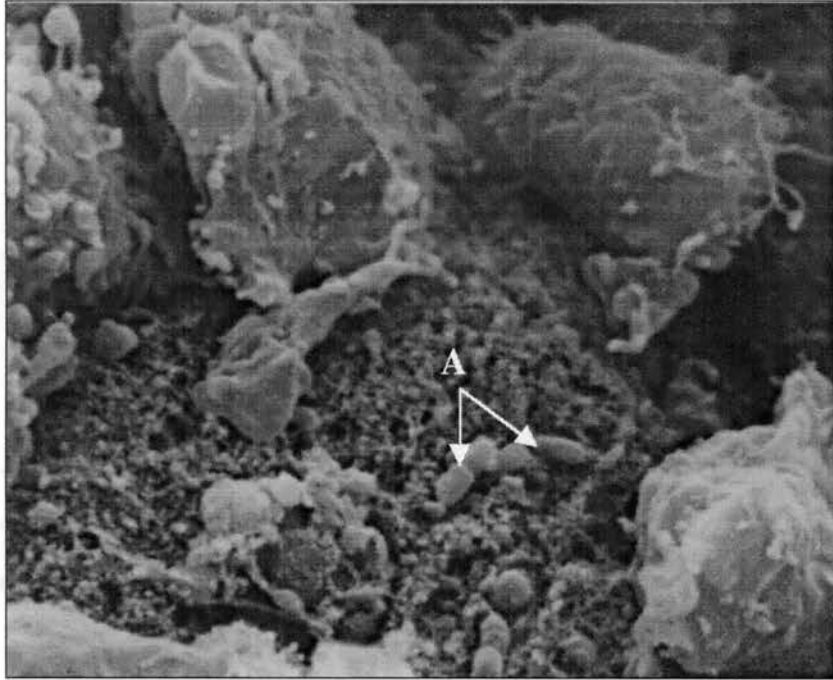
Figure 7. Scanning electron micrograph of bovine nasopharyngeal tissue after incubation with IBR-1 virus for 24 hours followed by incubation with *P. haemolytica* A1 for 18 hours. Note the presence of a few bacteria (A) adhered to an extensively damaged epithelium with absence of cilia (6000 X).

25 —

20 —

**

*

15 —

Mean % Adherence

10 —

5 —

0 —

3 hours    18 hours

0  1:10$^2$  1:10$^3$  1:10$^4$    0  1:10$^2$  1:10$^3$  1:10$^4$

**IBR Virus dilutions**
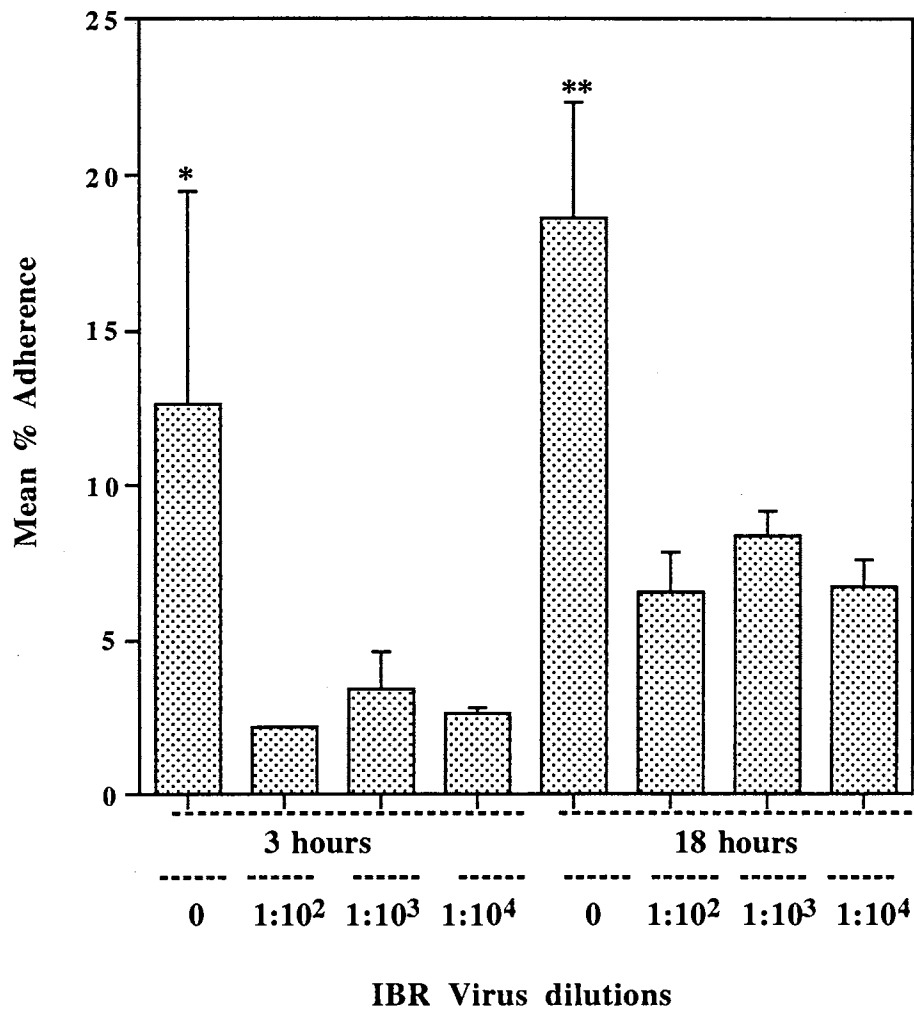
Figure 8.  Adherence of PhA1-668 to bovine nasopharyngeal tissue at 3 and 18 hours, after 24-hour preincubation with no virus or various dilutions of bovine herpes virus-1 (IBR virus).  Prepared nasopharyngeal tissue was incubated at 37C with 50 ul of either 0, 1:100, 1:1000, or 1:10,000 dilutions of IBR virus ($10^6$ $TCID_{50}$/ml) for 24 hours.  Viral-infected media was aspirated, tissue washed once with PBS, and reincubated at 37C with 50 ul of capsulated PhA1-668. After 3 hours and 18 hours, tissue was washed and digested, and radioactivity (Cpm) measured.  Data represent the means ± SD (error bars) of three replicates at both incubation times after preincubation at each of four viral dilutions.  *, ** Significantly (P<0.05) greater than adherence of PhA1 after 3 hours and 18 hours incubation, respectively, on viral pretreated tissue.

CHAPTER VII

SUMMARY CONCLUSIONS

The fact that *Pasteurella haemolytica* A1 is the most common bacterial pathogen isolated from the majority of BRD cases, and that the presence of this organism directly and indirectly causes severe damage to bovine lungs, is undisputed. Many years of field studies and observations have also established the epidemiology of this disease complex. Despite this, knowledge regarding the initial pathogenesis of the disease particularly in the URT is sadly lacking and leaves many unanswered questions regarding why and how this particular organism becomes predominant, particularly as it is very rarely isolated from the URT of a healthy calf, whilst *P. haemolytica* serovar A2 can be more readily found. The hypothesis that *P. haemolytica* A1 enters the lungs because it multiplies to high enough numbers in the URT to become aerosolized, rests on the supposition that in order to multiply, this particular *P. haemolytica* serovar must first colonize the URT mucosa. This usually involves adherence, which implies that *P. haemolytica* A1 must possess the ability to take advantage of the host's compromised innate immune system due to viral infection or stress, and establish itself in the URT probably by the expression of adhesins. Unfortunately, no definitive adhesins have been found. This lack of knowledge is in part due to the the inadequacy of the models used for this type of study in the bovine.

Thus, in order to determine whether *P. haemolytica* A1 adheres to bovine URT epithelial tissue, and if so, where and how this may occur, a modification of a tissue

culture model was developed for use with bovine epithelial and URT cell lines and adherence assayed using fluorescein-labeled bacteria. Adequate incubation time for adherence was allowed, followed by washing to remove non-adherent bacteria, and then measuring fluorescence to determine the mean % adherence. Fluorescein-labeling eliminated some of the experimental error in tissue culture adherence assays using quantitative culturing, where there is for e.g. inadequate release of adherent bacteria from cells by addition of detergent, and where labor-intensity due to bacteriologic culture of lysate dilutions is high. These experiments showed that a PhA1 isolate from a bovine pneumonic lung adhered, and did so to a greater extent than PhA2, suggesting that the predominance of PhA1 over PhA2 during the early stages of BRD may be attributable to the ability of PhA1 to adhere more avidly to URT tissues. This adherence may be related to serovar-specific expression of bacterial adhesins which have yet to be definitively identified. A study done to compare the effect of capsulated and noncapsulated bacterial adherence, indicated that noncapsulated PhA1 and PhA2 adhered better than capsulated bacteria. Further studies looking at the effect of blocking of bacterial adherence by PhA1 and PhA2 outer membrane proteins, produced no clear results, although PhA1 OMPs appeared to cause some degree of blocking. However, this type of tissue culture assay has many drawbacks, not least of which is that cell types available are undifferentiated, nonpolarised, and often have surfaces distinct from their origin especially in terms of distribution and accessibility of receptors. This lack of provision of a normal URT mucosa with cilia and mucus, as well as inherent assay problems, such as nonspecific, random adherence of bacteria to surfaces not occupied by host cells, were enough of a problem to develop a better assay that more closely mimicked the *in vivo* situation.

The *ex vivo* model was developed using bovine URT mucosa, and because of the typical epithelial surface with cilia and mucus production, was far better suited to explore adherence of *P. haemolytica*. Adherence of PhA1 was found to be highest on bovine nasopharyngeal tissue, and therefore all studies were performed using this mucosal surface. To test various factors involved in adherence, this model utilized bovine nasopharyngeal tissue incubated for 3 hours with radiolabeled bacteria, before washing off nonadherent bacteria, removing and digesting tissue with adherent bacteria, and measuring radioactivity to determine mean % adherence. Adherence of PhA1 was found to be significantly greater than that of PhA2, with capsulated PhA1 adherence being greater than that of decapsulated PhA1 and that of capsulated and decapsulated PhA2. These results, plus the fact that blocking experiments reduced the amount of adherence, indicated that PhA1 capsule may play an initial role in adherence to either mucus or epithelial cells. These results were opposite to those seen with the *in vitro* fluorometric model, probably because of divergence of the tissue culture model from the *in vivo* situation. Comparison of adherence of different serovars confirmed that lung isolates of PhA1 and PhA6 adhered better than all nasal isolates of either PhA1, PhA6, or PhA2. The fact that PhA1 and PhA6 strains originally isolated from the lungs of animals that had died from pneumonic pasteurellosis adhered to a greater degree than strains and serovars isolated from the URT of living animals adds further credence to the probability that differences in virulence between *P. haemolytica* serovars and strains is related to their ability to adhere and colonize the URT. Capsule may play a role in this. The final experiment to test the effect of IBR virus on adherence, showed that viral damage caused no increase in adherence, indicating that the major role of IBR in BRD may be to

compromise the host immune system which would have been difficult to assess in this model.

Thus, studies conducted using these assays of adherence have confirmed that *P. haemolytica* adheres to bovine URT epithelium and have provided strong circumstantial evidence that the greater virulence of PhA1 strains isolated from lungs of pneumonic animals may be related to their ability to adhere to and colonize the URT. Preliminary experiments exploring the involvement of bacterial factors in adherence have suggested an important role of capsule. It is expected that the availability of these models will greatly facilitate future experiments designed to elucidate mechanisms of *P. haemolytica* adherence more definitively.

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VITA

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