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Pasteurella multocida and bovine respiratory disease

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Abstract

Pasteurella multocida is a pathogenic Gram-negative bacterium that has been classified into three subspecies, five capsular serogroups and 16 serotypes. *P. multocida* serogroup A isolates are bovine nasopharyngeal commensals, bovine pathogens and common isolates from bovine respiratory disease (BRD), both enzootic calf pneumonia of young dairy calves and shipping fever of weaned, stressed beef cattle. *P. multocida* A:3 is the most common serotype isolated from BRD, and these isolates have limited heterogeneity based on outer membrane protein (OMP) profiles and ribotyping. Development of *P. multocida*-induced pneumonia is associated with environmental and stress factors such as shipping, co-mingling, and overcrowding as well as concurrent or predisposing viral or bacterial infections. Lung lesions consist of an acute to subacute bronchopneumonia that may or may not have an associated pleuritis. Numerous virulence or potential virulence factors have been described for bovine respiratory isolates including adherence and colonization factors, iron-regulated and acquisition proteins, extracellular enzymes such as neuraminidase, lipopolysaccharide, polysaccharide capsule and a variety of OMPs. Immunity of cattle against respiratory pasteurellosis is poorly understood; however, high serum antibodies to OMPs appear to be important for enhancing resistance to the bacterium. Currently available *P. multocida* vaccines for use in cattle are predominately traditional bacterins and a live streptomycin-dependent mutant. The field efficacy of these vaccines is not well documented in the literature.

Keywords: *Pasteurella multocida*, cattle, shipping fever, enzootic pneumonia, immunity, pathogenesis

Basic taxonomy of *Pasteurella multocida*

The taxonomy and nomenclature of *Pasteurella* have undergone many changes since first isolated by Pasteur as the causative agent of fowl cholera in 1880 (Fajfar-Whetstone *et al*., 1995). Nearly a half century later (in 1929), bacteria with common biochemical and morphological features were grouped together as *Pasteurella septica* and thereafter (in 1939) as *Pasteurella multocida* (Weber *et al*., 1984; Fajfar-Whetstone *et al*., 1995). Later, Mutters *et al*. (1985) reclassified the members of the genus *Pasteurella* into 11 species including *P. multocida* within which three subspecies (subsp.) *multocida*, *septica* and *gallicida* were defined based on sugar (D-sorbitol and dulcitol) fermentation. Molecular techniques based on M13 core fingerprinting and α-glucosidase activity were also developed to better differentiate between *P. multocida* subsp. *multocida* and subsp. *septica* (Hunt Gerardo *et al*., 2001). *P. multocida* subsp. *multocida*, the most important pathogenic member of the genus *Pasteurella sensu stricto*, has a broad host spectrum that includes many wild and domestic animal species. *P. multocida* subsp. *gallicida* and *septica* are specifically isolated from fowl and domestic pets (cats and dogs), respectively (Mutters *et al*., 1985). To define isolates associated with tiger bites, a fourth subspecies (*tigris*) was recently proposed by Capitini *et al*. (2002) on the basis of phenotype and 16S rRNA gene sequence comparison.

The capsule structure and lipopolysaccharide (LPS) composition of *P. multocida* are the basis for the classification of the bacterium into serogroups and

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serotypes, respectively (Heddleston *et al.*, 1972; Rimler and Rhoades, 1989). Passive hemagglutination and gel diffusion precipitin tests (reviewed in Rimler and Rhoades, 1989; Confer, 1993) have been traditionally used to classify *P. multocida* into five capsular groups (A, B, D, E and F) and 16 somatic serotypes (1–16), respectively (Carter, 1955; Heddleston and Rebers, 1972). Serogroups A and, to a lesser extent, D cause fowl cholera in birds (Rhoades and Rimler, 1989). Serogroup F strains are predominantly isolated from diseased poultry, in particular, turkeys and more recently from a fatal case of fibrinous peritonitis in calves (Shewen and Conlon, 1993; Catry *et al.*, 2005). In pigs, atrophic rhinitis and pneumonia are associated primarily with toxigenic strains of serogroup D and serogroup A, respectively (Dungworth, 1985), while *P. multocida* serogroups B and E are associated with hemorrhagic septicemia in cattle and water buffaloes in tropical regions of Africa and Asia (Carter and de Alwis, 1989; Rimler and Rhoades, 1989; Shewen and Conlon, 1993). In North America, serogroups B and E are rarely isolated from cattle (Confer, 1993). Worldwide, *P. multocida* serogroup A isolates are one of the major causes of bovine respiratory diseases (BRDs) (Frank, 1989; Rimler and Rhoades, 1989).

P. multocida association with BRD

It is clear that *P. multocida* is one of the primary bacterial pathogens associated with the clinical syndromes defining BRD complex including neonatal calf (enzootic) pneumonia and beef cattle pneumonia (shipping fever) (Lillie, 1974; Watts *et al.*, 1994; Fulton *et al.*, 2000; Welsh *et al.*, 2004). Both syndromes are considered multifactorial; that is, multiple infectious and non-infectious factors are involved in development of disease (Ames, 1997; Mosier, 1997). Unfortunately, many epidemiological studies of these conditions failed to differentiate among the variety of pathogens involved.

Strong evidence exists to link *P. multocida* to respiratory disease in dairy calves. This includes culture from lung tissue (Van Donkersgoed *et al.*, 1993; Sivula *et al.*, 1996a; Singer *et al.*, 1998; Hirose *et al.*, 2003), from nasopharyngeal swabs (NPS) (Catry *et al.*, 2006) and from transtracheal washes (TTW) (Singer *et al.*, 1998; Virtala *et al.*, 2000). The significance of *P. multocida* in calf pneumonia is more apparent than with shipping fever. Multiple studies involving dairy calves have identified *P. multocida* in much higher prevalence than *Mannheimia haemolytica* (Bryson *et al.*, 1978; Virtala *et al.*, 2000; Nikunen *et al.*, 2007). Studies conducted by Nikunen *et al.* (2007) on 84 calves with BRD in Finland found a significant relationship between the presence of *P. multocida* in calves with clinical signs of respiratory disease and the elevated concentrations of serum acute phase proteins (i.e. fibrinogen, haptoglobin, serum amyloid-A, LPS-binding protein and α1-acid glycoprotein), suggesting a strong pathogenic role for *P. multocida* in respiratory disease of calves. No significant relationship was observed for other pathogens including *Mycoplasma* sp., which were isolated in over 90% of calves as compared to 14% for *P. multocida*. Recent examination of 396 calves from 280 different dairy farms for occurrence of bacterial, mycoplasmal and viral pathogens found that *P. multocida* was the most commonly isolated bacterial pathogen and was detected in 26.4, 32.6 and 42.3% of tracheobronchial lavage samples from healthy, suspected or diseased calves, respectively (Autio *et al.*, 2007). Seventy-five percent of the *P. multocida* isolates were indole-negative phenotypes, underlining the need to investigate the role of indole-negative *P. multocida* isolates in calf respiratory disease. Nonetheless, the presence of the bacterium does not equal disease, as seroconversion to *P. multocida* occurred in control calves, confirming the ubiquitous nature of the organism and the multifactorial nature of the disease (Virtala *et al.*, 1996).

Evidence for involvement of *P. multocida* in shipping fever has been obtained by several methods. The bacterium was isolated from lungs of fatal cases in feedlot cattle by numerous investigators (Watts *et al.*, 1994; Fulton *et al.*, 2000; Storz *et al.*, 2000a; Welsh *et al.*, 2004; Gagea *et al.*, 2006). Indeed, *M. haemolytica* is the most common isolated bacterium from shipping fever, but the proportion of fatal cases of respiratory disease in feedlot cattle attributable to *P. multocida* appears to be increasing (Welsh *et al.*, 2004). Potential causes for this shift include changes in virulence among the pathogens, efficacy of antimicrobial agents, and changes in identification and/or management of sick cattle (Welsh *et al.*, 2004). *P. multocida* was cultured from nasal or NPS, TTW and/or bronchoalveolar lavage (BAL) of sick animals (Allen *et al.*, 1991; DeRosa *et al.*, 2000; Fulton *et al.*, 2002). A positive correlation was found between the species of bacteria isolated from multiple sites (e.g. NPS and TTW) (DeRosa *et al.*, 2000). The presence of *P. multocida* in BAL fluid accounted for ∼40% of respiratory disease (Allen *et al.*, 1992a). However, the presence or absence of *P. multocida* in the nasal pharynx did predict disease (Allen *et al.*, 1991; Fulton *et al.*, 2002). Similarly, seroconversion occurred in a high percentage of calves and thus was not predictive of disease (Virtala *et al.*, 2000). Nonetheless, low antibody concentration to *P. multocida* at feedlot entry correlated with decreased value to the owner (carcass value − total feedlot expenses) (Fulton *et al.*, 2002). Therefore, these various findings suggest that *P. multocida* is ubiquitous and can be found in both healthy and sick cattle; and not all calves harboring or exposed to the bacterium will develop pneumonia. However, its presence (particularly in large numbers) in the nares, trachea or bronchioles of a sick calf strongly suggests its involvement in the disease process.

Although knowledge of *P. multocida* serogroup and serotype specificity from BRD cases is limited, the

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reported data clearly singled out *P. multocida* serogroup A strains as the most frequently isolated from BRD cases (Blanco-Viera *et al.*, 1995; Ewers *et al.*, 2006; Autio *et al.*, 2007; Nikunen *et al.*, 2007). Studies by Blanco-Viera *et al.* (1995) showed that 61 and 25% of *P. multocida* isolated from pneumonic lung lesions belonged to capsular groups A and D, respectively, with serotype 3 (77%) being the most frequent. Kumar *et al.* (2004) investigated the prevalence of *P. multocida* serotypes in samples from different animal species in India. Cattle samples found to be positive for *P. multocida* were predominantly serotype A:3, in agreement with reports by Frank (1989). Studies in our laboratories found that 80% of *P. multocida* isolates from cattle with pneumonia were serotype A:3 (Dabo *et al.*, 1997), and recently, more than 92% of *P. multocida* isolated from cattle were found to be serogroup A (Ewers *et al.*, 2006).

Clinical disease: shipping fever

The term 'undifferentiated bovine respiratory disease' is often considered synonymous with 'shipping fever', although BRD may also be considered to include other disease entities, including enzootic calf pneumonia. For this discussion, respiratory disease in beef cattle will be referred to as shipping fever, which affects post-weaning beef calves, most commonly after transport to a stocker or feedlot operation. Diagnostic criteria are often vague, but typically include depression, inappetence, cough, nasal discharge and fever (or some combination of these). Most epidemiologic investigations relied upon producer treatment as a proxy for diagnosis. This frequently is no more exclusive than 'animals that are pulled for treatment and found to have no signs referable to a system other than the respiratory tract' (Bagley *et al.*, 2003; Cusack, 2004). Studies have shown limited correlation between treatment and more objective measures such as mortality, BAL cytology and lung lesions at slaughter (Allen *et al.*, 1992a, b; Griffin *et al.*, 1994; Ribble *et al.*, 1995a; Wittum *et al.*, 1996; Bryant *et al.*, 1999). Clearly the limitations of field-based diagnosis contribute significantly to the difficulty in studying shipping fever (Kelly and Janzen, 1986).

Morbidity and mortality due to shipping fever average approximately 14 and 1%, respectively (USDA National Animal Health Monitoring System (NAHMS)). However, both vary widely, with reported ranges for morbidity from 4.6% (Snowder *et al.*, 2006) to 89% (Storz *et al.*, 2000b) and for mortality from 1% (Snowder *et al.*, 2006) to 13% (Storz *et al.*, 2000b). Outbreaks are common, with initial cases appearing 7–10 days after arrival and typically peaking prior to 20 days (Jensen *et al.*, 1976; Alexander *et al.*, 1989). Mortality appears shortly after initiation of the outbreak, peaking at 16 days (Ribble *et al.*, 1995b). However, this timeframe can be lengthened by continued introduction of new animals into the herd or pens (Alexander *et al.*, 1989). Although disease occurs throughout the year, shipping fever has seasonal variation, peaking in fall to winter (Jensen *et al.*, 1976; Ribble *et al.*, 1995a; Loneragan *et al.*, 2001).

Numerous predisposing factors are frequently cited for shipping fever. The most well documented of these are viral infections, which will be discussed in regard to their specific association with *P. multocida*. Other proposed factors are generally termed 'stressors' and include transportation, co-mingling with other cattle, dust, cold, sudden and extreme changes of temperature and humidity, dehydration, hypoxia, endotoxin, cold coupled with wetness, and acute metabolic disturbances (Lillie, 1974; Irwin *et al.*, 1979). These are thought to alter the respiratory mucosa or hinder cattle's immune system, either directly or through the effects of endogenous agents such as cortisol, making the animals more susceptible to opportunistic infections. The intuitive nature of these suggestions is appealing, and they have become widely accepted and repeated throughout the literature. However, limited epidemiologic investigations have been done to confirm or refute the significance of these factors, and studies done thus far have been inconclusive. The most consistent finding in epidemiologic studies of shipping fever is the immense variability in occurrence, from operation to operation and year to year. This makes interpretation of interventions difficult, particularly in multi-year studies.

Several predisposing factors have been conclusively demonstrated. Newly received cattle are at greater risk for disease (Jensen *et al.*, 1976; Kelly and Janzen, 1986; Martin and Meek, 1986; MacVean *et al.*, 1986; Alexander *et al.*, 1989; Ribble *et al.*, 1995b). Calves purchased through a salebarn are more at risk than those arriving directly from farm sources (Wilson *et al.*, 1985; Gummow and Mapham, 2000). However, it is possible that other factors commonly associated with salebarn calves are responsible for the increased risk, rather than the salebarn process itself. These may include co-mingling of cattle from multiple sources, as well as the stress of repeated unloading, loading, handling, etc. Marketing through a salebarn may also reflect different management practices (e.g. better managers market direct, while poorer managers rely upon the salebarn). When examining what strategies can prevent disease, metaphylaxis (administration of parenteral antimicrobials to calves that are at high risk for respiratory disease) has consistently reduced morbidity (Van Donkersgoed, 1992; Frank *et al.*, 2000, 2002; Macartney *et al.*, 2003; Cusack, 2004), while results of vaccination have been equivocal at best (Mosier *et al.*, 1989; Perino and Hunsaker, 1997).

Clinical disease: enzootic calf pneumonia (ECP)

Similar to shipping fever, the term 'enzootic calf pneumonia' (ECP) is more a description than a diagnosis. In some ways, the distinction between ECP and shipping

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fever is arbitrary, with ECP occurring prior to shipping fever and after weaning. However, pneumonia is infrequent in pre-weaned beef calves on pasture. Thus, ECP usually refers to respiratory disease in dairy and veal calves raised in confinement. Diagnostic criteria for ECP are similar to those for shipping fever, both in content and vague nature. Studies have shown relatively poor correlation between producer-diagnosed ECP and that detected by a veterinarian (Van Donkersgoed *et al.*, 1993; Virtala *et al.*, 1999; Svensson *et al.*, 2006). Nevertheless, most field studies relied upon treatment by the producer as a proxy for disease, greatly complicating the conclusions that can be reached from such research.

ECP has traditionally been defined as developing between 2 and 6 months of age (Ames, 1997). Studies looking at younger calves found peak occurrence from nearly 4 weeks to 6 weeks of age (Waltner-Toews *et al.*, 1986; Curtis *et al.*, 1988a; Van Donkersgoed *et al.*, 1993; Virtala *et al.*, 1996). Reported morbidity varies significantly. Two studies found 6–8% of calves affected (Curtis *et al.*, 1988a; Sivula *et al.*, 1996b), whereas two other studies found morbidity rates of 15% (Waltner-Toews *et al.*, 1986) to 29% (Van Donkersgoed *et al.*, 1993). Mortality ranged from approximately 5% (Waltner-Toews *et al.*, 1986) to nearly 10% (Sivula *et al.*, 1996b).

Fewer studies have been done on the epidemiology of ECP compared to shipping fever. Waltner-Toews *et al.* (1986) and Van Donkersgoed *et al.* (1993) found larger herds had higher incidence of ECP than smaller herds. This relationship, however, was not identified by Curtis *et al.* (1988b). None of these studies included farms that would be considered large by today's standards (Waltner-Toews *et al.*, 1986). Waltner-Toews *et al.* (1986) and others (Bryson *et al.*, 1978; Svensson *et al.*, 2006) found a higher incidence in fall and winter but Curtis *et al.* (1988b) did not. Calves that had diarrheal disease may be at an increased risk for ECP (Curtis *et al.*, 1988b; Van Donkersgoed *et al.*, 1993; Svensson *et al.*, 2006). However, Van Donkersgoed *et al.* (1993) found that relationship was no longer significant after adjusting for clustering, and other studies did not identify a relationship between scours and respiratory disease (Virtala *et al.*, 1999). Failure of passive transfer (FPT) is an intuitive potential predisposing factor, but investigations of a possible relationship between FPT and ECP yielded conflicting results (Van Donkersgoed *et al.*, 1993; Virtala *et al.*, 1996, 1999). The diagnostic criteria for FPT may account for part of the discrepancies. Virtala *et al.* (1999) determined that immunoglobulin of less than 1200 mg dl^{-1} provides the best prediction of disease and thus the best break point for FPT. In contrast, Van Donkersgoed *et al.* (1993), Sivula *et al.* (1996a) and Virtala *et al.*, (1996) used values less than 800 mg dl^{-1} for diagnosing FPT. Another potential confounder is the diagnosis of respiratory disease. Van Donkersgoed *et al.* (1993) found no correlation between FPT and owner-diagnosed respiratory disease but found a positive association with that diagnosed by a veterinarian. Prado *et al.* (2006) found colostral antibodies to *P. multocida* to be short-lived, largely being undetectable by 2 months of age. Thus, their impact on ECP would likely be minimal.

Housing of calves was a significant influence on ECP in several studies. Several studies (Martin and Meek, 1986; Waltner-Toews *et al.*, 1986; Virtala *et al.*, 1999) found hutches to be better than inside pens, whereas Svensson *et al.* (2006) found housing calves individually better than group housing. Sivula *et al.* (1996a) were not able to demonstrate a reduction in ECP attributable to housing in hutches. Using the same data set, the authors applied objective criteria to define 'adequate ventilation of calf housing' and assessed its relationship with ECP (Sivula *et al.*, 1996b). There was a positive correlation between adequate ventilation of housing and average daily gain, but no relationship between either of these and ECP. These findings again could reflect the inadequacy of producer-diagnosed respiratory disease. Such an assertion is bolstered by the finding that producer-diagnosed death due to pneumonia only had a 58.3% sensitivity (Sivula *et al.*, 1996b). Numerous other factors have been potentially implicated in ECP. These include sire, vaccination of dam prior to calving, calves born in loose housing, time of day when born, how and when colostrum is provided, navel treatment, weaning based on size rather than age, use of non-medicated milk replacer, and provision of water and mineral.

Pathology of *P. multocida* pneumonia in cattle

Bovine pneumonia associated with *P. multocida* infections whether in young dairy calves, in weaned and shipped beef cattle or in calves experimentally challenged is often difficult to discern from pneumonia associated with other bovine bacterial pathogens such as *M. haemolytica* and *Histophilus somni*. Dungworth (Dungworth, 1985) indicated that 'less fulminating ... bronchopneumonias tend to be more often caused by *P. multocida* than by *P. [Mannheimia] haemolytica*'. In fact, isolation of more than one of these pathogens from pneumonic lungs in conjunction with respiratory viruses and/or *Mycoplasma* spp. occurs frequently (Gagea *et al.*, 2006). This has led authors to question if *P. multocida* is a primary pathogen in bovine pneumonia (Lopez, 2007). Experimental reproduction of bovine pneumonia with *P. multocida* alone and its isolation from cases of naturally acquired bovine pneumonia without evidence of other infectious agents indicate that it can likely occur as a primary pathogen in young or stressed cattle.

The gross pulmonary pathological changes have been described differently by various authors, and those designations probably reflect the age of the lesion, whether or not other infectious agents were involved but not identified, and descriptive preferences of the various authors. The lesion is a typical cranioventral

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bronchopneumonia and has been characterized simply as bronchopneumonia by some authors (Haritani *et al.*, 1989; Mathy *et al.*, 2002; Ewers *et al.*, 2006; Lopez, 2007) or as a bronchopneumonia with various descriptive modifiers denoting lesion age and type of exudate. These include acute fibrinosuppurative (Gagea *et al.*, 2006), subacute to chronic fibrinopurulent (Mosier, 1997), fibrinous to fibrinopurulent (Dungworth, 1985), suppurative (Tegtmeier *et al.*, 1999) and fibrino-necrotizing (Tegtmeier *et al.*, 1999). The presence of fibrinous to fibrinopurulent pleuritis, distended interlobular septa with edema or fibrin and/or abscesses is variable with *P. multocida* infection. Lopez (2007) designated the *P. multocida*-associated lesion as bronchointerstitial to bronchopneumonia depending on whether or not there was concurrent viral infection.

Histologic changes typically consist of bronchi and bronchioles filled with neutrophils, macrophages and necrotic epithelium interspersed with a small amount of fibrin (Dungworth, 1985; Jericho and Carter, 1985; Haritani *et al.*, 1989; Dowling *et al.*, 2004; Lopez, 2007). Neutrophil morphology ranges from intact to necrotic. Similar exudate is present in alveoli with peribronchiolar alveoli most severely affected particularly in the early phases of infection. The character of the exudate varies with time with acute lesions having a higher percentage of neutrophils and lower percentage of macrophages compared to chronic lesions. There is usually a variable amount of intra-alveolar hemorrhage, and *P. multocida* cells and antigen can be detected within alveolar lumens and within neutrophils and macrophages. Both intact and degenerating bacteria are seen within phagosomes of phagocytes (Haritani *et al.*, 1989), and intracellular survival of *P. multocida* within phagocytes has been demonstrated (Dowling *et al.*, 2004). Interlobular lymphatics can be dilated with fibrinous to fibrinopurulent exudate. Large foci of necrosis and abscesses have been described occasionally after experimental challenge (Dowling *et al.*, 2002; Mathy *et al.*, 2002; Ishiguro *et al.*, 2005). Immunohistochemical studies of natural cases of bovine pneumonia demonstrated, however, that abscesses were more often associated with *M. haemolytica*, *Arcanobacterium pyogenes* or *Mycoplasma bovis* infections and not with *P. multocida* (Haritani *et al.*, 1990; Adegboye *et al.*, 1995; Gagea *et al.*, 2006).

Epidemiology of *P. multocida* pneumonia

True epidemiologic investigation of *P. multocida*-associated respiratory disease is scant. One reason for this is the ubiquitous nature of the organism. Its mere presence in the upper respiratory tract is not diagnostic of disease; whereas confirmation of its involvement in pneumonia requires post-mortem culture or invasive measures (TTW or BAL), which are not practical for large numbers of cattle. It appears that *P. multocida* has a synergistic relationship with *Mycoplasma* species. This was particularly clear for dairy calf pneumonia, as isolation of both from clinical cases is more common than isolating either alone (Virtala *et al.*, 2000; Hirose *et al.*, 2003). Co-infection with other pathogens has also been noted, including *H. somni* (Gagea *et al.*, 2006), *M. haemolytica* (Van Donkersgoed *et al.*, 1993; Fulton *et al.*, 2000), coronavirus (Storz *et al.*, 2000a; Autio *et al.*, 2007), adenovirus (Autio *et al.*, 2007), parainfluenza-3 virus and bovine respiratory syncytial virus (Van Donkersgoed *et al.*, 1993; Autio *et al.*, 2007). The ability of *P. multocida* to cause disease in the absence of other pathogens has been debated (Bryson *et al.*, 1978). In a Finnish study, *P. multocida* was one of only three pathogens to be isolated more commonly from BAL of diseased than from healthy and suspect cattle (Autio *et al.*, 2007). However, no association was found between the isolation of *P. multocida* and clinical disease when no other pathogen was present (Autio *et al.*, 2007). This led them to accept the conclusion of Maheswaran *et al.* (2002) that *P. multocida* is an opportunistic pathogen and not a primary causative agent. In contrast, another Finnish study found *P. multocida* to be the only agent positively associated with clinical signs and elevated serum acute phase proteins (Nikunen *et al.*, 2007). Those authors concluded that there is 'a strong pathogenic role for *P. multocida* in respiratory disease in calves in Finland, at least in situations where other known pathogens are absent'. Finland is free of bovine viral diarrhea virus and *M. bovis*; therefore, findings there may not reflect disease where interaction between *P. multocida* and those agents may occur.

A major epidemiologic question is whether there is an inherent difference in pathogenic versus commensal populations of *P. multocida* or whether *P. multocida*-associated disease is strictly a change in host–bacteria relationship. Bacteriological characterizations have not identified an inherent difference between pathogen and commensal isolates, but the ability to detect such differences may be the limiting factor. Davies *et al.* (2003a) found that different subpopulations are responsible for progressive atrophic rhinitis and pneumonia in swine. Similarly, *P. multocida* isolates from ovine respiratory and reproductive tracts were often distinguishable (Davies *et al.*, 2003b). Therefore, differences (perhaps quite subtle) may exist between commensals and pathogens.

The entire *P. multocida* genome has now been sequenced, albeit from a single avian isolate, Pm70 (May *et al.*, 2001). This should enable relatively rapid characterization of genetic differences among various isolates and has in fact already yielded valuable information. Several virulence factors have been identified and are discussed elsewhere in this article. This section will focus only on the efforts to use bacterial products or genes encoding these products as markers for epidemiologic investigation. Hunt *et al.* (2001) identified several

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genes whose expression is up-regulated *in vivo*, including membrane proteins and metabolic and biosynthetic pathways. However, those were derived from bacteria inoculated into the bloodstream of mice and may not reflect events occurring in the upper or lower respiratory tract of healthy or sick cattle. Additionally, only one isolate of *P. multocida* was used; thus no comparative analysis was possible to determine what genes may promote virulence. Boyce and Adler (2006) concluded that 'true virulence genes might be constitutively expressed, upregulated only during initial stages of infection'. Thus, assaying for expression of virulence factors may be confounded by the progression of disease and interaction of host and pathogen. A more relevant comparative study examined the genotypes of a wide range of isolates from multiple host species (cattle, sheep and goats, bison, swine, rabbits, poultry, cats, dogs and humans), with some of the isolates being from diseased animals and others from clinically normal ones (Ewers *et al.*, 2006). They identified several genes that were more common in bovine isolates than from isolates obtained from other species. They also had modest success in identifying genes more commonly associated with isolates from diseased animals.

Another question relates to transmission, i.e. is *P. multocida* pneumonia contagious? The ubiquitous presence and commensal nature of *P. multocida* means that finding the organism in multiple animals does not demonstrate transmission. Instead, to assess transmission requires a way of discriminating between bacterial isolates. Classic means of distinction, including serotyping and serogrouping, are inadequate (Wilson *et al.*, 1992). Purdy *et al.* (1997) employed an extensive assessment of enzymatic activity to compare 61 *P. multocida* isolates collected from multiple locations over the course of 8 years. The profiles that resulted showed no evidence of clustering, suggesting that this practice offered little discriminatory power (Purdy *et al.*, 1997).

Another phenotypic approach to the question of transmission is assessment of antimicrobial resistance. Catry *et al.* found tetracycline-resistant *P. multocida* in the nasopharynx of 5 calves on 1 farm, while no resistant *P. multocida* were found in the other 56 calves from 15 farms (Catry *et al.*, 2006). No tetracycline had been used on any farms. The authors suggested that the five isolates were clonal, indicating horizontal transmission. A large retrospective study found evidence of regional differences in antimicrobial resistance among *P. multocida* isolates, with clustering in specific regions (Singer *et al.*, 1998). No further examination was done to assess whether isolates were clonal or even if the mechanism of antimicrobial resistance was consistent. A smaller study examined the genotypic basis for tetracycline resistance (Kehrenberg *et al.*, 2005). Six *Pasteurella* and *Mannheimia* isolates were obtained from a single farm where oxytetracycline had been given prophylactically. The same gene encoding resistance was found in multiple bacterial species, with little or no mutation. The authors concluded that *Pasteurella* and *Mannheimia* likely had been transmitted between calves. However, they acknowledged that horizontal transmission of resistance genes could also account for the findings. Because the same genetic material was found in multiple species of bacteria, it is possible that only the genes were passed from animal to animal (within another microbial species) and were subsequently taken up by the resident bacteria.

Genotypic analysis should be more insightful than the phenotypic methods described above for understanding transmissibility of *P. multocida*. Several methods have been used to examine isolates from several host species reviewed in Hunt *et al.* (2000). Molecular techniques that have been successfully used include restriction endonuclease analysis (REA), ribotyping, sequencing of rRNA, random amplification of polymorphic DNA (PCR fingerprinting), pulse field gel electrophoresis (PFGE) and multilocus sequence analysis as well as using PCR for capsular typing and analysis for the presence of the gene coding for dermonecrotoxin. Each of these techniques has both positive attributes and limitations: PFGE is considered the 'gold standard' of molecular epidemiology (Olive and Bean, 1999); however, few researchers have employed it in examining *P. multocida*, perhaps due to its time-consuming nature and relatively high equipment costs. Ribotyping and rRNA sequencing are less sensitive than other approaches in identifying differences among *P. multocida* isolates (Davies, 2004; Dziva *et al.*, 2004), whereas numerous researchers have employed PCR fingerprinting for epidemiologic investigation of *P. multocida* in several animal species (Chaslus-Dancla *et al.*, 1996; Zucker *et al.*, 1996; Dabo *et al.*, 1999a, b, 2000; Dziva *et al.*, 2001, 2004). Due to the relative low cost and ease of conducting PCR fingerprinting, this is arguably the technique of choice.

Most studies have used a combination of the above approaches and/or cellular characterization methods (whole cell protein profiles, outer membrane protein (OMP) profile, etc.). Davies *et al.* (2003a) used OMP profiles, capsular typing and the presence of dermonecrotoxin to compare *P. multocida* isolates from porcine pneumonia and found limited diversity among isolates. They offered two potential explanations: either there is little diversity among the commensal population of *P. multocida* with little potential for diversity in disease state or there are a few virulent clones that account for disease under most circumstances. Similar techniques (capsular typing and OMP profiles) using avian isolates, however, demonstrated wide diversity of strains, suggesting that avian disease more likely represents opportunistic infection (Davies *et al.*, 2003b).

Davies *et al.* (2003a) also examined bovine isolates, using a combination of multilocus sequence analysis, OMP profiling and rRNA sequencing. They found only a limited number of OMP types predominated among bovine isolates, even from isolates obtained from diverse

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geographical regions. They, therefore, speculated that few clones have a marked capacity to cause disease (similar to the proposed situation in swine). Using ribotyping, we (Dabo *et al*., 1999a) found little heterogeneity among isolates from diverse geographical areas, but the significance of this is uncertain considering the limitations of ribotyping. All of Davies isolates were from diseased animals, whereas we used isolates from healthy and diseased cattle. While we (Dabo *et al*., 1999a) did not identify which isolates were obtained from healthy versus sick animals, some variation was found in ribotypes of isolates obtained from lung which were from diseased animals compared to nasal passage, which were potentially from healthy or diseased animals. This could be consistent with differences existing between commensal and pathogenic isolates. DeRosa *et al*. (2000) found only one ribotype among *P. multocida* isolates obtained from both TTW and NPS of sick calves. They concluded that this method is not sensitive enough to discern relatedness. However, given the findings of others (Dabo *et al*., 1999a; Davies *et al*., 2003a), it is possible that all isolates in DeRosa's population were clonal in origin. DeRosa also performed antibiograms to compare isolates obtained from the two sources (TTW or NPS). The authors state that 'although a few paired isolates exhibited identical ribotypes, their antibiotic susceptibility profiles were different'. However, the study included both *P. multocida* and *M. haemolytica*, and there is no clarification of which species demonstrated the divergent ribotyping and antibiogram results. When taken together, these findings indicate that an inherent difference exists between commensals and pathogens. It is clear, though, that additional work is required to confirm this, preferably using a consistent methodology with a more sensitive molecular approach. This final point is reinforced by several studies that found different grouping patterns among the same isolates when using multiple molecular or cellular techniques (Dabo *et al*., 1999a; Blackall *et al*., 2000; Davies, 2004; Davies *et al*., 2004).

The primary involvement of *P. multocida* in BRD is well established. Nonetheless, the understanding of the disease entities and the bacterial mechanisms in causing the diseases is still rudimentary. Improved methods of diagnosis of shipping fever and ECP will likely be needed before progress can be made in clarifying the host and environmental factors that contribute to the diseases. In contrast, it is possible that the tools needed for understanding the pathogen's role already exist. Hopefully there will soon be an answer as to what (if anything) distinguishes the commensal *P. multocida* from that which causes disease.

P. multocida virulence factors

The potential *P. multocida* virulence factors and features of the bacterium important in BRD infection are limited. The review below will focus on the virulence factors associated with *P. multocida* serotype A isolates.

Adherence and colonization factors

Attachment is a primary prerequisite for bacterial infections of a host, and ligands that are involved in such adherence are potential virulence factors. Several studies examined the adherence of different *P. multocida* isolates to different cell types, tissues or organs and reflected serogroup-specific host preference and pathogenicity (Glorioso *et al*., 1982; Jacques *et al*., 1988; Ackermann *et al*., 1991; Letellier *et al*., 1991; Dugal *et al*., 1992; Esslinger *et al*., 1994; Isaacson and Trigo, 1995; Al-Haddawi *et al*., 2000; Borrathybay *et al*., 2003; Ali *et al*., 2004a). Fimbriae, as adherence mediators in *P. multocida* serotype A, have been reported (Glorioso *et al*., 1982; Rebers *et al*., 1988; Ruffolo *et al*., 1997). *P. multocida* serotype A specifically bound HeLa and rabbit pharyngeal cells *in vivo* and *in vitro* with fimbriae as putative adhesins (Glorioso *et al*., 1982). *P. multocida* serogroup A, B, D and B:2 strains are known to possess type IV fimbriae (designated PtfA) (Ruffolo *et al*., 1997; Doughty *et al*., 2000; Siju *et al*., 2007), and sequence comparison revealed variation within the gene among isolates (Doughty *et al*., 2000). However, the role of type IV fimbriae in *P. multocida* serotype A adherence to host cells has not been reported. A 39 kDa OMP, lipoprotein B (plpB) from *P. multocida* serotype A isolates was shown to be an adherence factor and stimulated cross-protective immunity in mice and poultry (Borrathybay *et al*., 2003; Ali *et al*., 2004a, b; Tabatabai and Zehr, 2004; Harper *et al*., 2006).

The *P. multocida* genome contains several adhesin-related genes including homologs of *Bordetella pertussis* filamentous hemagglutinin (pfhaB1 and pfhaB2) and *Heamophilus influenzae* surface fibril (Hsf1 and Hsf2) proteins (May *et al*., 2001). Application of SPAAN (Sachdeva *et al*., 2005) to *P. multocida* whole genome revealed several uncharacterized novel adhesions. *B. pertussis* FHA family of proteins are known or suspected to play a crucial role in bacterial adherence to and colonization of host cells (Barenkamp and St Geme, 1994; Barenkamp, 1996; Barenkamp and St Geme, 1996a, b; Ward *et al*., 1998). We previously cloned and sequenced in bovine *P. multocida* strains a gene encoding a protein related to *B. pertussis* FHA proteins (Dabo and Confer, 2001; PmFHA, GenBank accession no. AY035342). *H. influenzae* type b Hsf is a major vitronectin-binding protein that contributes to serum resistance of that bacterium (Hallstrom *et al*., 2006). Both *P. multocida* FHA and Hsf proteins were found to be transcriptionally activated under nutrient-deficient conditions (Paustian *et al*., 2002). Additionally, signature-tagged mutagenesis (SMT) identified four adhesin-related genes in bovine *P. multocida* strains that attenuate

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Table 1. Potential sites for *Pasteurella multocida* adherence in various diseases and lesions

Lesion	Animal species (Disease)	Adherence sites
Upper and lower respiratory disease	• Cattle (Shipping fever or neonatal dairy calf) • Swine bronchopneumonia • Swine atrophic rhinitis • Rabbits (Snuffles) • Birds (Fowl cholera)	• Upper respiratory mucosa • Lower respiratory mucosa • Pneumocytes • Alveolar interstitical ECM • Upper respiratory ECM
Septicemia	• Cattle (Hemorrhagic septicemia) • Rabbits (Septicemic snuffles) • Birds (Septicemic fowl cholera)	• ECM • Endothelium • ECM

ECM, extracellular matrix.

virulence in a murine septicemia model (Fuller *et al.*, 2000); these include the two *pfhaB1* and *pfhaB2* genes, one gene (*pfhaC*) that is similar to hemolysin accessory gene of *B. pertussis FhaC*, and one gene (*tadD*), a homolog of *Actinobacillus actinomycetemcomitans tadD* protein (Fuller *et al.*, 2000). *TadB* is part of *flp* (fimbria-like protein) operon, a widespread locus in bacteria known to be essential for virulence in *A. actinomycetemcomitans* and *Haemophilus ducreyi* (Kachlany *et al.*, 2000, 2001). The expression of *P. multocida tad* genes was also up-regulated under starvation conditions (Paustian *et al.*, 2001). Studies by Tatum *et al.* (2005) showed that *P. multocida pfhaB2* mutant was highly attenuated in turkeys following intranasal challenge, suggesting a role in initial colonization by *P. multocida*; however, *pfhaB2* mutant and parent strain were both resistant to complement killing. Recently, Ewers *et al.* (2006) showed that only 46% of the cattle isolates investigated harbored the *fha* gene, and a significant association was reported between disease status and bovine *P. multocida* strains harboring *pfhA* gene. Hypothetically, *P. multocida* FHA may also function as a serum resistance factor based on sequence homology to *H. somni* immunoglobulin-binding p76 protein (Cole *et al.*, 1993). A related immunoglobulin-binding protein (designated Pm76) was previously identified and sequenced in bovine *P. multocida* strains (Dabo and Confer, 2001–PmFHA, GenBank accession no. AAK61596).

The mechanisms of *P. multocida* adherence (not colonization) to bovine cells or the bacterial factors involved in such adherence in BRD infections have been poorly addressed. Many pathogens including members of the family *Pasteurellaceae* exploit host extracellular matrix (ECM) molecules for virulence through adherence and colonization (Duensing and van Putten, 1997, 1998; Molinari *et al.*, 1997; Duensing *et al.*, 1999; Bresser *et al.*, 2000). The broad host range characteristic of *P. multocida* suggests recognition of host cell surface components (ECM molecules) common to multiple animal species and tissue types (Table 1). Recent studies in our laboratories investigated the binding of *P. multocida* to ECM molecules and demonstrated that bovine *P. multocida* isolates bind to several ECM molecules including fibronectin (Fn) (Dabo *et al.*, 2005). Bacteria bound both soluble and immobilized Fn and preferentially bound the N-terminal heparin-binding fragment (Hep-1) of Fn, similar to that described for other bacterial pathogens (Westerlund and Korhonen, 1993; Patti *et al.*, 1994; Joh *et al.*, 1999; Dabo *et al.*, 2005). The *P. multocida* proteins identified as putative Fn-binding proteins include *P. multocida* OmpA, the TonB-dependent receptor HgbA (hemoglobin-binding protein A), the transferrin-binding protein A (TbpA) and Pm10769 (Dabo *et al.*, 2003, 2005). *P. multocida* OmpA bound Madin–Darby bovine kidney (MDBK) via heparin and/or Fn bridging (Dabo *et al.*, 2003), and monoclonal antibodies against OmpA significantly reduced or abolished bacterial binding to and invasion of MDBK cells (S. M. Dabo and A. W. Confer, unpublished data). The *OmpA* gene from bovine *P. multocida* strains exists as an eight-stranded transmembrane anti-parallel β-barrel that displays four surface-exposed loops with putative roles in adherence and serum resistance (Dabo *et al.*, 2003). In *Escherichia coli*, OmpA has been associated with virulence, functions as a multifunctional protein displaying phage receptor and porin activities (Datta *et al.*, 1977; Schweizer and Henning, 1997), is involved in the bacterial invasion of host cells (Prasadarao, 2002; Prasadarao *et al.*, 1996) and contributes to serum resistance (Weiser and Gotschlich, 1991).

Two *P. multocida* regulatory genes have also been reported as potential virulence factors: DNA adenine methylase (*Dam*) and the Rci recombinase (*rci*) gene (Fuller *et al.*, 2000; Chen *et al.*, 2003). In *Salmonella* Typhi, Rci protein inverted the DNA in the C-terminal region of the type IV pili, resulting in the inhibition of the minor pilus protein expression and the promotion of bacterial self-association (Morris *et al.*, 2003; Tam *et al.*, 2004). Whether Rci of *P. multocida* has a similar action on the bacterium type IV pili is unknown. The *dam* gene regulated the expression of virulence genes in several Gram-negative bacteria including *P. multocida* (Blyn *et al.*, 1990; Chen *et al.*, 2003; Heusipp *et al.*, 2007). Overexpression or deletion of *dam* gene in several organisms resulted in attenuation for mice (Heithoff *et al.*, 1999; Chen *et al.*, 2003; Badie *et al.*, 2007). *P. multocida* serotype A harboring a plasmid-mediated alteration of *dam* expression was highly attenuated in mice (Chen *et al.*, 2003). However, a *Klebsiella pneumoniae dam* mutant showed only partial attenuation in mice (Mehling *et al.*, 2007).

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Neuraminidase (sialidase) is prevalent among *P. multocida* strains and is expressed *in vivo* by *P. multocida* A:3 strains associated with bovine pneumonia (White *et al.*, 1995; Straus *et al.*, 1996; Ewers *et al.*, 2006); both cell-bound and extracellular sialidases were reported (White *et al.*, 1995). Its role in BRD, however, remains largely unknown. Evidence of neuraminidase function in *P. multocida* disease suggested that the protein is protective in mice (Ifeanyi and Bailie, 1992), is associated with virulence (Muller and Krasemann, 1974) and may contribute to bacterial adherence, colonization and persistence (Mizan *et al.*, 2000). However, the relationship between the ability of *P. multocida* to produce the enzyme and to cause pneumonia in cattle has not been established. Two sialidase genes (*nanB* and *nanH*) were reported in 100 and 88.5% of *P. multocida* bovine strains investigated, respectively (Mizan *et al.*, 2000). Mutation in *nanH* resulted in a bacterium that was not deficient in sialidase production but had reduced enzyme activity (Mizan *et al.*, 2000). As reported by Steenbergen *et al.* (2005) and reviewed by Harper *et al.* (2006), Pm70 genome database contains several putative sialometabolic gene products, and the uptake, not catabolism, of sialic acid appeared essential for virulence in mice by protecting bacteria from innate host defense mechanisms that may promote persistence (Steenbergen *et al.*, 2005; Harper *et al.*, 2006).

In summary, determining the *P. multocida* genes involved in bacterial adherence, primarily during the early stage of infection, can assist in our understanding of the molecular pathogenesis of *P. multocida*. Changes in host–pathogen interaction resulting from changes in environmental signals are most likely the cause of differences between commensal and pathogenic *P. multocida*. The molecular differences between commensal and pathogenic *P. multocida* are not at present understood, but the mechanisms clearly involve the ability of the latter to induce inflammatory responses most likely through toll-like receptor (TLR) signaling. TLR signaling is crucial to innate host defense, which provides the first line of defense against infection through bacterial identification and killing and activation of adaptive immunity. Because a growing number of bacterial adhesins are reported to depend on TLR for induction of proinflammatory cytokines (Abramson *et al.*, 2001; Hajishengallis *et al.*, 2002; Ogawa *et al.*, 2002; Lorenz *et al.*, 2004; Inatsuka *et al.*, 2005; Fischer *et al.*, 2006), future studies should focus on the identification of proinflammatory *P. multocida* adhesins as potential vaccine candidates.

LPS

P. multocida LPSs have similar properties (biological and chemical) and structure to R-type LPS (with no polymeric O-antigen) found in many Gram-negative bacteria (Lugtenberg *et al.*, 1984; Rimler, 1990; Confer, 1993; Harper *et al.*, 2006). These molecules induce classic signs of endotoxic shock (Rimler and Rhoades, 1989). The LPS structure has been determined for three *P. multocida* serotype A strains (VP161, X73 and Pm70) (St Michael *et al.*, 2005a, b, c) and revealed a highly conserved inner but variable outer core (Harper *et al.*, 2006). The investigation of the *P. multocida* Pm70 genome also identified gene clusters that may be responsible for LPS biosynthesis (St Michael *et al.*, 2005c). Pathogenic bacteria expressing similar LPS include *H. influenzae*, *Campylobacter jejuni*, and *Neisseria* spp., and changes in the outer core oligosaccharides of these bacteria are often the result of phase-variation strategies in key LPS genes (Raetz and Whitfield, 2002). No such evidence was found in LPS genes in the *P. multocida* genome (Harper *et al.*, 2007). Clusters of several putative glycosyltransferase genes, not always found in this genus, were identified in *P. multocida* Pm70 genome (May *et al.*, 2001; St Michael *et al.*, 2005c).

A novel finding of *P. multocida* LPS is the identification of two predicted heptosyl-1-transferases (designated *HptA* and *HptB*) expressing a single or two Kdo residue(s), respectively (St Michael *et al.*, 2005a). *hptA* is annotated as *opsX* in *P. multocida* Pm70 genome for its homology to the same gene in *H. influenzae*, and *hptB* is a homolog to *waaC* of *E. coli* and *K. pneumoniae* (St Michael *et al.*, 2005a; Harper *et al.*, 2007). Virulence studies show that the *hptB* mutant was fully virulent (Harper *et al.*, 2007), whereas the *hptA* mutant was fully attenuated (Harper *et al.*, 2004, 2007). Therefore, *hptA* may be critical for the survival of the organism in the host.

Purified *P. multocida* LPS is highly antigenic and capable of producing widespread vascular alterations and death, but it is poorly immunogenic (Heddleston and Rebers, 1975; Tsuji and Matsumoto, 1988; Ryu and Kim, 2000). Generally, *P. multocida* LPS is immunogenic or protective only when complexed with proteins (Ganfield *et al.*, 1976; Tsuji and Matsumoto, 1988; Ryu and Kim, 2000) or ribosomes (Phillips and Rimler, 1984). Ryu and Kim (2000) demonstrated complete protection in mice vaccinated with *P. multocida* type A:3 LPS–protein complex; however, neither the LPS or protein alone nor the LPS and protein as a mixture provided protection. The authors also showed that the LPS–protein complex induced both humoral and cell-mediated immunity (Ryu and Kim, 2000). Previously, a bactericidal monoclonal antibody against LPS from a *P. multocida* serotype A isolate completely protected mice against homologous challenge (Wijewardana *et al.*, 1990; Confer, 1993). Sutherland *et al.* (1993) also found that polyclonal anti-idiotype antibodies that mimic a linear *P. multocida* LPS molecule protected mice against homologous challenge (Sutherland *et al.*, 1993). However, studies by Lu *et al.* (1991) failed to show protection in mice against *P. multocida* infection following passive immunization with affinity-purified rabbit anti-LPS serum. An opsonic

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but not bactericidal anti-LPS monoclonal antibody only partially protected against *P. multocida* infection in mice (Ramdani and Adler, 1991; Confer, 1993).

LPS is also known as a stimulator of host defense against *P. multocida* infection (Iovane *et al.*, 1998; Galdiero *et al.*, 2000). Purified *P. multocida* LPS stimulated the expression and release of several cytokines in mice including the inflammatory (IL-1α, IL-6 and TNF-α) and immunoregulatory cytokines (IFN-γ and IL-12), which are known to play an important role in host defense (Iovane *et al.*, 1998). Galdiero *et al.* (2000) also demonstrated a role for *P. multocida* LPS in neutrophil adhesion and migration through bovine endothelial cell monolayers. In another study, LPS from a *P. multocida* serotype A strain enhanced humoral and cell-mediated immune responses in chickens (Maslog *et al.*, 1999).

Capsule and the capsule biosynthetic genes

The genetic organization of the capsule biosynthetic loci from the five *P. multocida* serogroups was determined and reviewed (Chung *et al.*, 1998; Boyce *et al.*, 2000a; Townsend *et al.*, 2001; Harper *et al.*, 2006). Comparative analysis of the five capsular biosynthetic regions identified serogroup-specific regions confirming a genetic basis for the serological differences between the observed strains (Carter, 1952; Rimler and Rhoades, 1989; Townsend *et al.*, 2001).

The importance of capsule as virulence determinants in the pathogenesis of *P. multocida* has been clearly established; however, the focus to date has been primarily on *P. multocida* capsular types A and B from fowl cholera and hemorrhagic septicemia infections, respectively (reviewed in Harper *et al.*, 2006) (Boyce and Adler, 2000; Chung *et al.*, 2001; Harper *et al.*, 2006). Studies on the specific role of bovine *P. multocida* capsule in BRD infections are non-existent. Virulence studies in mice indicated that isogenic serotype A mutants were attenuated as compared to their encapsulated wild types (Jacques *et al.*, 1993; Boyce and Adler, 2000; Chung *et al.*, 2001). Transposon insertion into a hyaluronan synthase gene of bovine *P. multocida* strain attenuated virulence in a septicemic mouse model (Fuller *et al.*, 2000). Watt *et al.* (2003) reported the loss of virulence of unencapsulated variants of a *P. multocida* serotype A strain and the restoration of virulence with the capsulated phenotype in mice. The capsular dissociation was not due to alteration in capsular gene nor was it regulated by the two-component (histidine kinase/response) regulator of capsule synthesis which was absent in *P. multocida* isolates under investigation as well as the Pm70 genome database.

Although *P. multocida* capsules are implicated in the virulence of *P. multocida*, vaccination of mice with capsule did not stimulate protection (Esslinger *et al.*, 1993, 1994; Gutierrez-Pabello *et al.*, 1995; Pruimboom *et al.*, 1996). In general, many virulent strains of *P. multocida* express capsule consisting of hyaluronic acid (HA) (types A and B), chondroitin (type F) or heparin (type D) (Carter and Chengappa, 1980; Muniandy and Mukkur, 1993; Boyce *et al.*, 2000b; DeAngelis and Padgett-McCue, 2000; DeAngelis and White, 2002; DeAngelis *et al.*, 1998, 2002) that are similar to molecules found naturally in the various hosts (Snipes *et al.*, 1987; Rimler and Rhoades, 1989; Tsuji and Matsumoto, 1989; DeAngelis, 1996). This 'molecular mimicry' prevents the mounting of a strong antibody response to capsular materials, impairs phagocytosis, reduces the action of complement killing and increases adherence to and survival in the host (Harmon *et al.*, 1991). Paradoxically, only *P. multocida* capsular B strains, and more specifically B:2 strains, produced hyaluronidase, an enzyme that specifically cleaves HA between its repeating disaccharides (*N*-acetylglucosamine and D-glucuronic acid) (Carter and Chengappa, 1980; Rimler and Rhoades, 1994; Harper *et al.*, 2006).

Antiphagocytic activity of *P. multocida* was reported and associated with the presence and thickness of capsule (Truscott and Hirsh, 1988; Harmon *et al.*, 1991; Pruimboom *et al.*, 1996). Decapsulation of *P. multocida* serotype A:3 with hyaluronidase increased phagocytosis by macrophages, whereas an unencapsulated variant of the bacteria was not internalized (Pruimboom *et al.*, 1996). Moreover, isogenic non-encapsulated *P. multocida* serotype A strains had significantly increased sensitivity to phagocytosis than the encapsulated wild-type strain (Boyce and Adler, 2000). Serum resistance correlated with encapsulation in *P. multocida* serotype A, because spontaneous, isogenic acapsular mutants or decapsulated strains were generally sensitive to complement-mediated killing compared to wild-type strains (Snipes and Hirsh, 1986; Hansen and Hirsh, 1989; Chung *et al.*, 2001). In contrast, acapsular *P. multocida* B2 mutants were resistant to complement-mediated killing (Boyce and Adler, 2000).

Conflicting reports exist with regard to the role of *P. multocida* capsule in adherence, which may be dependent both on the bacterial strain and on the type of host cells used in the studies. Pruimboom found that *P. multocida* adhesion to macrophages was mediated by capsular HA (Pruimboom *et al.*, 1996). Enzymatic depolymerization of the capsule decreased *P. multocida* serotype A adherence to HeLa cells and macrophages (Esslinger *et al.*, 1994). Reduced adherence to macrophages was reported for a spontaneous *P. multocida* serotype A mutant (Pruimboom *et al.*, 1996). *P. multocida* serotype A strains also had reduced adherence to peripheral blood monocytes as compared to air sac macrophages, and the exposure of monocytes to anti-HA-binding cell surface proteoglycan (CD44) monoclonal antibody decreased bacterial binding (Pruimboom *et al.*, 1999). In contrast, Glorioso *et al.* (1982) showed increased adherence of *P. multocida* serogroup A to HeLa cells and rabbit pharyngeal cells following enzymatic

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depolymerization of the capsule. Others demonstrated that the absence of capsule increases *P. multocida* adherence to and colonization of lung and tracheal by cells (Jacques *et al.*, 1994).

OMPs

Several *P. multocida* OMPs possess virulence attributes including surface exposure, *in vivo* expression, immunogenic and bactericidal properties (Confer, 1993; Confer *et al.*, 1996; Marandi and Mittal, 1996; Dabo *et al.*, 1997; Vasfi Marandi and Mittal, 1997; Gatto *et al.*, 2002; Borrathybay *et al.*, 2003; Davies *et al.*, 2004). Our studies of bovine *P. multocida* serogroup A isolates identified several surface-exposed and *in vivo*-expressed OMPs, including an *E. coli* OmpA and a *H. influenzae* type b (Hib) P1 homolog proteins (Dabo *et al.*, 1997). *P. multocida* OmpA is a major immunogenic and antigenic OMP that is conserved, surface-exposed and expressed *in vivo* (Dabo *et al.*, 1997, 2003). High antibody responses to this and several other OMPs consistently correlated with resistance of cattle to challenge with virulent *P. multocida* (Dabo *et al.*, 1997, 2003).

OmpH is another major antigenic, surface-exposed and conserved OMP porin that is detected in 100% of bovine isolates investigated (Lugtenberg *et al.*, 1984, 1986; Chevalier *et al.*, 1993; Ewers *et al.*, 2006) and has potential as a vaccine candidate (Luo *et al.*, 1997, 1999; Vasfi Marandi and Mittal, 1997). The protein provided protection against *P. multocida* challenge in mice (Vasfi Marandi and Mittal, 1997) and chickens (Luo *et al.*, 1997, 1999), and its expression was shown to be negatively regulated by iron and glucose. Electrophoretic analysis showed that OmpH levels increase in *P. multocida fur* mutant compared to wild-type strain (Bosch *et al.*, 2001). The expression of OmpH–lacZ fusion in the wild-type strain increases in the presence of chelating agent 2,2′-dipyridyl (DPD), and there was a 5-fold decline in the expression of the gene in the wild type as compared to the *fur* mutant when grown in the presence of glucose. Given the importance of OmpH as a potential immunogen and the observed inhibitory effect on OmpH expression, those authors recommended growing bacteria in the absence of glucose for bacterin production (Bosch *et al.*, 2001).

Several *in vivo* studies identified potential *P. multocida* virulence genes and OMP-associated cross-protection factors (Rimler, 1994; Wang and Glisson, 1994a, b; Hunt *et al.*, 2001). When using antisera produced against live *P. multocida* isolates, Wang and Glisson (1994a, b) found four high-molecular-weight proteins (ranging from 204 to 153 kDa) that stimulate cross-protection. Hunt *et al.* (2001) used *in vivo* expression technology (IVET) to identify two lipoproteins (PM0554 and PM14440) with homology to conserved and widely distributed PCP (peptidoglycan-associated lipoprotein (Pal) cross-reaction protein), and protein D of non-typeable *H. influenzae*, respectively. The *H. influenzae* protein D mediates the binding to IgD, a form of immune evasion, and stimulates cross-protection against heterologous challenge in rats (Akkoyunlu *et al.*, 1996). Anti-PCP serum was bactericidal against *H. influenzae* clinical isolates (Deich *et al.*, 1990). *P. multocida* PCP was found to be surface-exposed, while protein D (designated GlpQ) was not (Lo *et al.*, 2004); however, neither protein stimulated protection against challenge with live *P. multocida* in mice and chickens. Boyce *et al.* (2006) found significant up-regulation of Pm0803 and the ef-Tu (elongation factor-Tu) during growth *in vivo* and significant down-regulation of OmpW. ef-Tu (tufA and tufB) is cytoplasmic in location but may be targeted to the outer membrane since its homologs bind host fibronectin in *Mycoplasma pneumoniae* and host mucin in *Lactobacillus johnsonii* (Boyce *et al.*, 2006). While the major *P. multocida* surface proteins have been studied, little is known about the less abundant proteins and their roles in the pathogenesis of *P. multocida* infections in general.

Iron-regulated and acquisition proteins – IROMPs

Iron acquisition and uptake are essential for bacterial survival and pathogenic bacteria have developed different strategies for that uptake. *P. multocida* produces both iron-chelating siderophores and outer membrane receptors for the iron-binding host molecules (transferrin, hemoglobin and hemin) (Choi-Kim *et al.*, 1991; Ogunnariwo *et al.*, 1991). Early studies showed that some *P. multocida* isolates produced siderophores (Choi-Kim *et al.*, 1991) or contained only one specific Tbp (TbpA) (Ogunnariwo and Schryvers, 2001; Ogunnariwo *et al.*, 1991). *P. multocida* grown under iron-depleted media or *in vivo* expressed three iron-regulated OMPs (IROMPs) with molecular masses of 76, 84 and 94 kDa, with all three having affinity for siderophore binding (Choi-Kim *et al.*, 1991).The *P. multocida tbpA* gene was absent in avian isolates and detected only in ruminants (cattle, 70%; sheep, 80%; and buffalo, 57.1%) (Ewers *et al.*, 2006). TbpA from *P. multocida* serotype B:2 strain was predicted to be a gated pore with several membrane-spanning regions (Shivachandra *et al.*, 2005). The specific role of TbpA in *P. multocida* virulence is still unknown.

Analysis of attenuated mutants by Fuller *et al.* (2000) identified two loci containing potential virulence genes with homology to iron-acquisition-related genes *ExbB* and *hgbA* of *H. influenzae*. *ExbB*, along with *exbD*, are part of the *tonB* operon in *P. multocida* and are independently transcribed (Bosch *et al.*, 2002a). In a related study, Bosch *et al.* (2002b) demonstrated that three ORFs (PM0298, PM0299 and PM300) of *P. multocida* constitute a single transcriptional operon. Mutants defective in *PM0298* or *PM0299* demonstrated that these genes were essential for the viability of the pathogen (Bosch

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et al., 2002b). The product of PM300 (designated HgbA) bound hemoglobin (Bosch *et al.*, 2002b) and hemin (Bosch *et al.*, 2004), but binding and virulence of the *hgbA*-mutant strain were not affected (Bosch *et al.*, 2002b). Similarly, insertional inactivation of *hgbB* did not affect the ability of *P. multocida* to bind hemoglobin (Cox *et al.*, 2003), suggesting the presence of additional hemoglobin receptor proteins in the *P. multocida* genome. Consistent with that finding, comparative analysis of the *P. multocida* Pm70 genome (May *et al.*, 2001) identified nine iron-acquisition-related genes (including *hgbA*) encoding immunogenic proteins (Bosch *et al.*, 2004). Six proteins bound both hemin and hemoglobin and the other two bound either hemin or hemoglobin. These proteins did not stimulate protective immunity in mice against *P. multocida* infection when inoculated alone (Bosch *et al.*, 2004). *P. multocida* HbpA (hemin-binding protein A), another iron acquisition gene, was shown to be immunogenic and regulated through a Fur-independent mechanism; HbpA-specific antibodies were not protective in mice (Garrido *et al.*, 2003). The prevalence of *tonB*, *tbpA*, *hgbA* and *hgbB* genes in *P. multocida* bovine strains is reported to be 100, 70.2, 95.2 and 57.7%, respectively (Ewers *et al.*, 2006), and there was significant association between disease status and the presence of *hgbB* and *tbpA* as single virulence-associated genes in *P. multocida* bovine isolates. Our characterization of a heme acquisition system receptor (HasR) in a *P. multocida* bovine isolate demonstrated that the protein, which is 98% identical to its homolog in the *P. multocida* Pm70 genome sequence, is surface-exposed, is conserved among most *P. multocida* isolates and is an immunodominant IROMP (Prado *et al.*, 2005). Finally, a recent comparative transcriptional response to iron limitation between *M. haemolytica* and *P. multocida* found few homologous genes induced in both organisms and include the hemoglobin receptor *hmbR2*, the *fbpAB, yfeABCD ABC*-type systems, *tonB* and *exbD* (Roehrig *et al.*, 2007). The hemin and hemoglobin transporter genes were among the genes uniquely found up-regulated in *P. multocida*, an indication of its capacity to utilize iron from blood sources (Roehrig *et al.*, 2007).

Extracellular enzymes

Several extracellular enzymes such as proteases and lipases have also been reported as possible virulence factors in *P. multocida* (Negrete-Abascal *et al.*, 1999; Pratt *et al.*, 2000). The role of lipases in pathogenic bacteria is probably nutritional, whereas proteases may assist pathogens against host defense degrading host IgG and reducing opsonization. Negrete-Abascal (1999) reported the secretion of proteases into the culture supernatant of *P. multocida* from different species including bovine. Those proteases were capable of degrading host IgG in a wide range of pH (Negrete-Abascal *et al.*, 1999). Pratt *et al.* (2000) demonstrated the production of lipases by clinical isolates of *P. multocida* from different species. The role, if any, these enzymes play in the pathogenesis of *P. multocida* infections is unknown.

Immunity against *P. multocida* serogroup A in cattle

Immunity of cattle to *P. multocida* has been investigated most extensively with respect to hemorrhagic septicemia of cattle and buffalo involving serotypes B:2 and E:2 (Confer, 1993). Studies on immunity in cattle pneumonia caused by *P. multocida* serotype A isolates are limited. This section of the review will focus on current knowledge of bovine immunity against *P. multocida* serotype A isolates.

Important antigens responsible for immunity against *P. multocida* serotype A isolates in cattle are not completely understood. Adler *et al.* (1999) reviewed candidate vaccine antigens and genes in *P. multocida* related to various diseases of production animals and indicated that LPS can provide some protection against homologous serotypes. They further reviewed several putative immunologically important antigens by focusing on studies that focused on antigens expressed under *in vivo* conditions. These include OMP Oma87, which is a homologue of the D15 protective antigen of *H. influenzae*; type 4 fimbria and its subunit protein PtfA; capsule; and IROMPs, such as the Tbp TbpA. None of these putative vaccine candidates were directly investigated with respect to stimulating immunity in cattle against *P. multocida* serotype A. The gene for Oma87 has been cloned and expressed; however, vaccination against that protein failed to protect chickens against an experimental challenge (Mitchison *et al.*, 2000; Chaudhuri and Goswami, 2001). The *ptfA* gene was demonstrated in most bovine *P. multocida* isolates and has been cloned (Adler *et al.*, 1999; Ewers *et al.*, 2006); however, immunogenicity was not reported. The potential for TbpA to be a good vaccine candidate is questionable, because approximately 30% of bovine *P. multocida* strains lack the *tbpA* gene (Ewers *et al.*, 2006; Roehrig *et al.*, 2007). We suggested that OMPs from bovine *P. multocida* serotype A strains may be good vaccine candidates based on correlations between high antibodies and resistance against experimental bovine *P. multocida*-induced pneumonia (Confer *et al.*, 1996). However, Abdullahi *et al.* (1990), using bovine *P. multocida* serotype A isolates, questioned the potential efficacy of an OMP vaccine based on data obtained from vaccination of mice with whole bacterial cell vaccines followed by intraperitoneal challenge.

Immunity in bovine respiratory pasteurellosis is presumed to be antibody-mediated. Anti-*P. multocida* antibodies were demonstrated in dairy cow colostrum by quantitative indirect immunofluorescence and

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agglutination assays (Gresham *et al.*, 1984). Passive antibodies to *P. multocida* serotype A were demonstrated in sera from newborn dairy and beef calves, and those passively acquired antibodies were short-lived in sera compared to values of 4–6 months for antiviral antibodies demonstrated in one of these studies (Virtala *et al.*, 1996; Fulton *et al.*, 2004; Step *et al.*, 2005; Prado *et al.*, 2006). Virtala *et al.* (1996) found no correlation between postcolostral antibody titers in dairy calves with and without naturally acquired respiratory disease, usually associated with *P. multocida* and/or *Mycoplasma dispar* infection. Passively acquired anti-*P. multocida* OMP antibodies were demonstrated in dairy calf sera within 24 h of birth (Fulton *et al.*, 2004; Step *et al.*, 2005). Step *et al.* (2005) demonstrated that while dairy calves were isolated in hutches, *P. multocida* antibody concentrations declined to a nadir between 47 and 73 days of age. Those calves were subsequently removed from hutches, grouped together and spontaneously seroconverted to *P. multocida*, most likely from exposure to the bacterium from other calves and from fomites or from stress-related factors stimulating commensal *P. multocida* proliferation. In beef calves, similar passive/acquired anti-*P. multocida* antibody responses were found in calves from two different herds (Prado *et al.*, 2006). Serum concentrations of anti-*P. multocida* antibodies in neonatal calves varied greatly between the two herds. In both herds, however, passively acquired IgG1 antibodies declined rapidly and reached their nadir between 60 and 90 days of age. This was followed by a rapid rise in anti-*P. multocida* IgM and IgG2 antibodies that reached peak concentrations between 150 and 180 days of age. Although, at this time, protection against respiratory disease has not been associated with passively acquired anti-*P. multocida* antibodies, their short-lived nature negates any potentially protective role after approximately 3 months of age.

As indicated above, active antibody responses to *P. multocida* develop in calves at a young age at least as measured by ELISA (Virtala *et al.*, 1996; Step *et al.*, 2005; Prado *et al.*, 2006). Whether antibodies acquired naturally or from vaccination truly protect cattle against *P. multocida*-associated pneumonia is not well documented. Serum anti-*P. multocida* antibody concentrations were associated with several cattle production parameters in a study of cattle shipped from 24 different ranches to a feedlot without going through the order buyer/sale barn marketing system (Fulton *et al.*, 2002). In that study, high antibodies to *P. multocida* OMPs at the time of feedlot entry were associated with increased average daily gain during feeding, whereas low *P. multocida* antibodies were associated with a decreased net value to owner, decreased gross margin and increased total costs of finishing. There was, however, no association between health or treatment costs and *P. multocida* antibody concentrations. Few of the represented herds had been vaccinated with *P. multocida* vaccines. Therefore, it is assumed that the antibodies measured were from prior natural exposure.

Enhanced resistance to respiratory *P. multocida* challenge has been demonstrated in several studies using live vaccines. These include wild-type bacteria, streptomycin-dependent mutant and chemically modified *P. multocida*. (Kucera *et al.*, 1981; Panciera *et al.*, 1984; Chengappa *et al.*, 1989; Confer *et al.*, 1996; Mathy *et al.*, 2002). In addition, Kadel *et al.* (1985) demonstrated greater weight gains, less severe clinical signs of pneumonia and lower death rates in calves vaccinated with both live streptomycin-dependent *P. multocida* and live streptomycin-dependent *M. haemolytica* when compared to non-vaccinated calves. In several studies, a correlation was demonstrated between high serum *P. multocida* antibodies and resistance against experimental challenge in live *P. multocida*-vaccinated cattle. Compared to controls, cattle vaccinated with live *P. multocida* via aerosol or subcutaneous routes had significant reduction in lesion scores (Panciera *et al.*, 1984) or reduced histologic lesions, reduced neutrophil influx, reduced IL8 and IL1β, and increased IL2 responses (Mathy *et al.*, 2002). Live *P. multocida*-vaccinated calves also developed serum antibodies against the whole bacteria (Panciera *et al.*, 1984) or to outer membrane preparations (Confer *et al.*, 1996) using quantitative indirect immunofluorescence assay or ELISA, respectively. In both studies, there was a significant correlation ($P < 0.05$) between high antibodies against *P. multocida* and low lesion scores following transthoracic challenge. In addition, using Western blotting and densitometric analyses, high serum antibodies against six specific OMPs (approximately 100, 90, 85, 74, 53, 35 and 16 kDa) significantly correlated with less severe lesions following *P. multocida* challenge (Confer *et al.*, 1996). Amino-terminus sequencing demonstrated that the 35 kDa protein is a heat modifiable (28–35 kDa) OMP from the OmpA family of proteins (Dabo *et al.*, 1997); however, vaccination of mice with the partially purified protein did not protect against intraperitoneal *P. multocida* challenge (Gatto *et al.*, 2002). Another *P. multocida* OMP shown to be of importance in stimulating resistance to infection in animals is OmpH, an approximately 33.6–38.5 kDa protein (Vasfi Marandi and Mittal, 1997; Davies and Lee, 2004). That protein, however, has not been studied immunologically in cattle.

Studies of vaccination of cattle with non-living *P. multocida* vaccines have also been limited. Early studies suggested some protection of cattle against shipping fever using *Pasteurella* spp. bacterins (reviewed in Collins, 1977). Vaccination of calves with a vaccine containing modified-live- Parainfluenza 3 virus, killed *M. haemolytica* and killed *P. multocida* vaccine enhanced resistance against experimental challenge involving stress and aerosol exposure to all three infectious agents (Matsuoka *et al.*, 1966). In more recent years, however, neither aerosol nor intratracheal vaccination of calves

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with formalin-killed *P. multocida* resulted in protection against experimental pulmonary challenge (Confer *et al.*, 1996; Dowling *et al.*, 2004; Prado *et al.*, 2005). In our laboratory, we demonstrated that calves vaccinated with Freund's incomplete adjuvant–outer membrane preparations from *P. multocida* cultured in the presence or absence of an iron chelator (IROMPs or OMPs, respectively) developed antibodies to OMPs and IROMPs, as detected by ELISA (Prado *et al.*, 2005). Vaccination with OMPs or IROMPs resulted in significant decrease in lesion scores following *P. multocida* challenge compared to vaccination with adjuvant alone. Of specific interest was that vaccinated calves developed intense antibody responses to a 96 kDa protein band, and correlation between high anti-96 kDa antibodies and low lesion scores approached significance ($P < 0.06$). The 96 kDa OMP was a homologue to the iron-regulated protein HasR, a heme acquisition receptor protein. The *hasR* gene, however, was not demonstrated in all *P. multocida* isolates examined. Therefore, its potential immunological significance is not known.

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