IMMUNOLOGICAL CHARACTERIZATION OF IRON-REGULATED OUTER MEMBRANE PROTEINS OF *Pasteurella multocida* A:3 WITH EMPHASIS ON HasR

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INTRODUCTION

Introduction and review of relevant literature

Bovine respiratory disease (BRD) is the most economically significant syndrome affecting North American beef cattle. Reports from the National Animal Health Monitoring System (NAHMS) list respiratory disease as the leading cause of death (prevalence 28%), costing the cattle industry $ 478 million dollars per year (USDA, 1997). Furthermore, estimated annual losses due to BRD exceed $1 billion when other factors such as decreased productivity, treatment costs and labor are considered (Griffin, 1997).

The etiology of BRD is complex and to this day not well understood. Previous research has shown that BRD results from the interaction of different stressors such as intensive management practices, long transport, abrupt weather changes and viral infections, all of which can impair the host defense mechanisms and favor the colonization of the lower respiratory tract by bacterial pathogens such as *Mannheimia haemolytica*, *Pasteurella multocida*, *Haemophillus somnus* and *Mycoplasma* spp. (Dyer, 1982). Second to *M. haemolytica*, *P. multocida* group A type 3 (A:3) is most commonly isolated from cases of BRD (Dyer, 1982; Frank, 1989). *P. multocida* has been shown to produce sub acute to chronic suppurative bronchopneumonia in feedlot cattle and dairy calves that is less severe than the fulminating fibrinous pleuropneumonia caused by *M. haemolytica* (Mosier, 1997). Experimentally, respiratory disease may be reproduced by transthoracic or intratracheal inoculation of *P. multocida* A:3 in calves (Panciera and Corstvet, 1984; Chengappa *et al.*, 1989; Gourlay *et al.*, 1989; Dowling *et al.*, 2002; Mathy *et al.*, 2002).

Pathogenesis

The pathogenesis of *P. multocida* pneumonia is not well understood. Most of the information has been extrapolated from studies of *P. multocida* hemorrhagic septicemia, which is exotic to North America, and *M. haemolytica* pneumonia. *P. multocida* is a common inhabitant of the upper respiratory tract of healthy cattle, and under normal conditions cattle are able to prevent *P. multocida* infections (Dyer, 1982; Frank, 1989; De Alwis, 1992). However, proliferation of bacteria in the lungs of calves is favored when host defense mechanisms such as mucociliary clearance and alveolar macrophages are impaired. The mucociliary apparatus, comprised of ciliated and non-ciliated epithelial cells, goblet cells and bronchial mucous glands, covers most of the respiratory tract extending from inside the nostrils to the terminal bronchioles. The cilia on epithelial cells beat in an upward motion or "forward", propelling impinged bacteria and debris cranially. This type of motion allows the animal to clear the airway of foreign matter (Weekly and Veit, 1995). Respiratory viral infections can damage local host defense mechanisms, thus allowing bacteria to colonize and proliferate. A preceding infection with a respiratory virus(es) is of paramount importance in the development of *P. multocida* pneumonia. Infections with bovine herpesvirus-1 (BHV-1), parainfluenza-3 (PI-3) or bovine respiratory syncitial virus (BRSV) damage the mucosa of the respiratory tract, reducing pulmonary clearance and thus allowing bacteria to colonize and cause disease (Confer *et al.*, 1988; Baker *et al.*, 1997; Kapil and Basaraba, 1997; Potgieter, 1997). In addition, BHV-1 impairs macrophage, polymorphonuclear neutrophil (PMN) and lymphocyte functions (Kapil and Basaraba, 1997). Similarly, PI-3 affects alveolar macrophages resulting in decreased phagocytosis and killing of bacteria, and pulmonary

inflammation (Kapil and Basaraba, 1997). The direct role of bovine viral diarrhea virus (BVDV) in initiating respiratory tract disease is not well understood; however, it might be the result of its profound negative effects on systemic immunity and pulmonary resistance (Potgieter, 1997). Infection with bovine coronavirus (BCV) has been associated primarily with upper respiratory disease in cattle; however, it also has the potential for causing pneumonia (Kapil and Basaraba, 1997). In a recent study, BCV was implicated in two outbreaks of pneumonia in feedlot cattle, suggesting that this virus might also play a role in the development of BRD (Storz *et al.*, 2000).

Another factor that contributes to weakening of the host immune system is stress. Different stressors such as inhalation of high levels of irritant gases (truck exhaust and/or ammonia), abrupt changes in temperature and humidity, overcrowding, and shipping all produce similar results, allowing commensal bacteria, usually from the upper respiratory flora, to colonize the lower respiratory tract (Dyer, 1982). During processing, transport and commingling of cattle there is an increase in the release of endogenous steroids which may continue to be elevated for an extended period of time. Increased steroid levels for prolonged periods can have a detrimental effect on serum neutrophil and lymphocyte functions, decreasing bactericidal actions of phagocytes as well as cellular immunity (Simensen *et al.*, 1980; Dyer, 1982; Elenkov and Chrousos, 1999). The source of *P. multocida* that colonizes the lower respiratory tract (LRT) resulting in pneumonia appears to be the upper respiratory tract (URT). *P. multocida* can be found in the URT of healthy calves in low numbers (Frank and Smith, 1983). When cattle are subjected to stressful conditions such as those described above, there is proliferation of *P. multocida* in the URT, which results in aerosolation and inhalation of the bacteria to the lungs.

With impaired immune mechanisms and decreased capacity of the lung to remove this pathogen, inhaled *P. multocida* can colonize the LRT (Mosier, 1997).

Pasteurella multocida classification

P. multocida is a gram-negative non-motile, non-spore forming, small coccobacillus or rod (0.2 μm to 2.0 μm) that is a member of the family *Pasteurellaceae* pohl. Pasteurellaceae, which includes many pathogenic bacteria of the genera *Pasteurella, Haemophillus, Actinobacillus* and *Mannheimia*, have a worldwide distribution and are capable of affecting a broad range of species, producing diseases in humans and primarily in lower mammals and birds. Most *Pasteurellae* are commensals on the mucous membranes of the URT and intestinal tract of wild and domestic animals. *P. multocida* culture characteristics include fermentative, oxidase- and catalase-positive, and varying ability to cause hemolysis. This bacterium is a facultative anaerobe, growing well at 37°C with 5% CO_2. Growth occurs in unenriched media, but is enhanced with supplements of serum or blood. It does not grow on McConkey agar. Bacterial colonies, usually obvious by 24 hours, are of moderate size, round and grayish, and non-hemolytic with a sweetish odor.

P. multocida has been classified into 5 serogroups (A, B, D, E and F) based on the capsular polysaccharide antigen (CPS) (Carter, 1967; Rimler and Rhoades, 1987). The somatic antigen present on the lipopolysaccharide (LPS) molecule is the base for the classification of *P. multocida* into 16 serotypes (1-16) (Heddleston *et al.*, 1972). Additionally, *P. multocida* may be classified according to molecular characteristics found by polymerase chain reaction (PCR) fingerprinting, restriction endonuclease analysis (REA), ribotyping, and pulsed-field gel electrophoresis (PFGE) as reviewed by Hunt *et*

al. (2000). Recently, May *et. al.* (2001), determined the complete sequence of the genome of *P. multocida* strain Pm70, an avian A:3 strain. The genome consists of 2,257,487 base pairs encoding 2,014 open reading frames.

Virulence factors of *Pasteurella multocida*

During the last decade advances in molecular biological techniques have helped unravel and identify many virulence factors of bacterial pathogens including those of *P. multocida*. Yet, there are still many unknowns regarding the pathogenic mechanisms of this bacterium. So far the most important virulence factors identified for *P. multocida* include adhesins (fimbriae and filamentous haemagglutinin), sialidases, CPS, LPS, heat shock proteins, a toxin designated PMT (*Pasteurella multocida* toxin that is present in some strains), outer membrane proteins (OMPs), and iron-regulated outer membrane proteins (IROMPs) (Confer, 1993; Ruffolo *et al.*, 1997; Fuller *et al.*, 2000).

Adhesins

Adhesion of *P. multocida* to the mucosal surface of the URT would appear to be the first logical step for colonization and establishment of infection in the host. It is not known how *P. multocida* adheres to host cells; however, two types of adhesins might be involved. Adhesins have been shown to be essential for mediating adherence of other bacterial pathogens (Wizemann *et al.*, 1999). *P. multocida* encodes genes for filamentous hemagglutinin (FHA) and type 4 pili (TFP). The presence of the FHA gene has been demonstrated in bovine and avian isolates of *P. multocida* (Fuller *et al.*, 2000; May *et al.*, 2001). Fuller *et al.* (2000), identified two FHA genes (termed *pfhB1* and *pfhB2*) by signature-tagged mutagenesis (STM). The presence of these two genes was later confirmed by May *et al.* (2001), by complete genome sequencing of *P. multocida* strain

Pm70. These genes code for proteins with high homology to the filamentous hemagglutinin (FhaB) of *Bordetella pertussis*, which is known to be an important adhesin and immunogen of this pathogen.

Several aspects of *P. multocida* PfhB1 and PfhB2 protein sequences suggest that these two proteins might be associated with virulence (May *et al.*, 2001). For example, there is a conserved motif, determined by the following amino acid (aa) sequence N(P/Q)NG(I/M), in the N-terminal region that is typical of all members of this large FHA protein family. This motif is involved in post-translational processing and extracellular signaling. Further, there are several integrin-binding motifs in the central region, also a characteristic of this family of proteins, and their presence suggests that the PfhB1 and PfhB2 proteins may be involved in adherence of *P. multocida* to host cell surfaces. Finally, there is a region in the C-terminus that shares 66% homology with p76, a serum-resistance protein of *H. somnus*. This protein confers resistance against opsonization to *H. somnus*. PfhB2 also contains two 400-aa direct repeats like those found in p76. Accordingly, the PfhB proteins may well be a major virulence factor for *P. multocida,* and genetic manipulation of *pfhB* might allow for the development of sub-unit vaccines.

Type 4 fimbriae encoded by the *ptfA* gene have been identified and characterized in strains of *P. multocida* belonging to serogroups A, B and D (Ruffolo *et al.*, 1997; Doughty *et al.*, 2000). These types of fimbriae are long, filamentous appendages expressed on the surface of some gram-negative bacteria in which they are essential for attachment to host surfaces (Adler *et al.*, 1999). Characterization of these structures showed a high similarity with the N-terminal aa sequence of other type 4 fimbrial

subunits from *Pseudomonas aeruginosa, Dichelobacter nodosus, Moraxella bovis, H. influenzae* and *Neisseria gonorrhea* (Ruffolo *et al.*, 1997).

Sialidases

P. multocida produces sialidases, also known as neuraminidases, that can cleave the mucus present in the bovine URT and might promote adhesion to the mucosal surface. In general, sialidases release sialic acid from sialyl-conjugated glycoproteins, glycolipids, or colominic acids by cleaving alpha-ketosidic linkages (Mizan *et al.*, 2000). The role of sialidases in the disease process remains incompletely defined; however, they increase the virulence of several microorganisms such as *Vibrio cholerae, Haemophillus influenzae, Neisseria meningitides,* and *Streptococcus pneumoniae.* In the case of *V. cholerae,* sialidases act on the mucous layer, allowing the bacterium to gain access to gangliosides located on the epithelial cell surface (type G_{M1}) that function as receptors for cholera enterotoxin (Corfield, 1992). *H. influenzae* and *N. meningitides* express a sialyltransferase that adds sialic acid to their LPS structure, thereby mimicking host structures which promotes their survival by decreasing the bactericidal effects of complement (Shakhnovich *et al.*, 2002). In contrast, *S. pneumonia* produces sialidases that desialylate the cell surfaces of *N. meningitides* and *H. influenzae* making them more vulnerable to the bactericidal effects of complement thus allowing *S. pneumonia* to compete for the same host niches (Corfield, 1992; Shakhnovich *et al.*, 2002). Therefore, sialidases may be produced by bacterial pathogens to gain access to the host cell receptors as well as for removal of sialic acid attached to the cell surface of other bacteria. In addition, the enzyme is toxic to host tissues and interferes with host immunologic and defense mechanisms. Furthermore, a positive correlation has been

established between the level of production of sialidases by some bacterial strains and their virulence (Corfield, 1992).

The majority of *P. multocida* isolates express sialidases (Drzeniek *et al.*, 1972; Ifeanyi and Bailie, 1992; Straus *et al.*, 1996b). It was previously shown that a bovine *P. multocida* A:3 isolate produced both extracellular and cell-bound sialidases (White *et al.*, 1995). Subsequently, two sialidases encoded by the genes *nanH* and *nanB* were characterized for this bacterium from three different strains (Mizan *et al.*, 2000). The predicted molecular masses were 80 and 120 kDa, respectively. In addition, the N-terminal 400 aa of NanH has 50% similarity to the *Salmonella enterica* serotype Typhimurium and the small clostridial sialidases. In contrast, NanB had a predicted sequence of 510-aa and shares 50% homology with the *S. pneumoniae* sialidase (Mizan *et al.*, 2000). It was also suggested that NanB as well as NanH are potential members of an autotransporter family, a newly discovered system of protein secretion. However, further work is needed to characterize these genes and their products (Mizan *et al.*, 2000).

Polysaccharide Capsule

Most *P. multocida* isolates express a capsular polysaccharide on their surface. In general, capsules are highly hydrated polyanionic polysaccharides that are covalently linked to the cell surface by phospholipids or lipid A molecules (Roberts, 1996). Based on the capsular antigen, *P. multocida* isolates are classified into serogroups A, B, D, E and F. These serogroups have species/disease predilection, such that serogroups B and E are associated with haemorrhagic septicemia in bovidae, serogroup A with fowl cholerae in birds, and serogroup D with atrophic rhinitis in swine. The serogroup A:3 is the most commonly isolated serotype from cases of BRD in North America (Dabo *et al.*, 1999). In

contrast to other serogroups, the capsule of *P. multocida* A contains mainly hyaluronic acid, a polymer of D-glucuronic acid and N-acetyl-D-glucosamine (Carter and Annau, 1953; Rosner *et al.*, 1992; Pandit and Smith, 1993).

The function of the capsule in *P. multocida* has not been investigated; however, it most likely protects the bacterium from dehydration, which may enhance its survival during transmission from host to host or in the environment. Capsules promote adherence in other bacterial species (*Pseudomonas* spp.), while in the case of *P. multocida* there is conflicting evidence that varies with the strain of *P. multocida* and the type of cells used in the adherence assay (Boyce *et al.*, 2000). Nevertheless, *P. multocida* A isolates adhere strongly to HeLa cells, turkey air sac macrophages and alveolar macrophages (Esslinger *et al.*, 1994; Pruimboom *et al.*, 1996). Adhesion was shown to be reduced following treatment with hyaluronidase or when a spontaneous acapsular variant of *P. multocida* A was used in the adherence assays (Esslinger *et al.*, 1994; Pruimboom *et al.*, 1996). In contrast, Glorioso *et al.*, (1982) found that pretreatment of cells with hyluronidase increased the adherence of *P. multocida* A to HeLa cells and rabbit squamous pharyngeal cells, and that *P. multocida* D strains with reduced capsule had increased adhesion to porcine respiratory tract cells (Jacques *et al.*, 1993; Jacques *et al.*, 1994). Based on these findings, it was suggested that the capsule might interfere with some outer membrane structures in these strains (such as fimbriae or LPS) that might be involved in adherence (Jacques *et al.*, 1993; Jacques *et al.*, 1994).

The effect of *P. multocida* capsule on phagocytosis has been previously studied (Boyce and Adler, 2000, 2001; Chung *et al.*, 2001). The capsule provides the bacterium with a shielding effect, protecting the cell by minimizing its exposure to the host immune

system and complement factors. In consequence, capsules promote antiphagocytic activity and impair opsonization of bacteria by neutrophils or macrophages (Moxon and Kroll, 1990; Roberts, 1996). The exact mechanism for this interaction is unknown but could be explained by the effect that the negative charge of the capsular surface has on the interface between the cell and the phagocyte, resulting in impaired digestion (Moxon and Kroll, 1990; Boyce *et al.*, 2000). The amount of capsule and the intensity of the negative charge are directly proportional to the resistance of the bacteria to phagocytosis (Maheswaran and Thies, 1979; Moxon and Kroll, 1990; Harmon *et al.*, 1991). In addition, the amount of capsule on *P. multocida* can be decreased by growing the bacterium in the presence of iron chelators (Jacques *et al.*, 1994). Spontaneous and constructed acapsular mutants of different *P. multocida* strains or cells chemically decapsulated with hyaluronidase have been shown to be more sensitive to phagocytosis than encapsulated strains (Jacques *et al.*, 1993; Boyce and Adler, 2000).

Some encapsulated strains of *P. multocida*, particularly those of serogroup A, are highly resistant to bactericidal complement activity and readily grow in serum, whereas spontaneous acapsular mutants as well as cells chemically treated with hyaluronidase have increased sensitivity to complement-mediated killing (Snipes and Hirsh, 1986; Hansen and Hirsh, 1989). In contrast, there was no difference in sensitivity to complement-mediated killing between encapsulated and isogenic non-capsulated strains of *P. multocida* B:2 strains (Boyce and Adler, 2000).

The capsular biosynthetic loci for two strains of *P. multocida* (A:1 and B:2) have been determined, and like other gram-negative pathogens such as *H. influenzae*, they were found to be genetically organized into three distinct regions designated 1, 2 and 3

(Boulnois and Roberts, 1990; Chung *et al.*, 1998; Boyce and Adler, 2000; Boyce *et al.*, 2000). This information has allowed defined genetic manipulation of the capsule so that its role in virulence can be better determined.

Lipopolysaccharide

Like other gram-negative pathogens, *P. multocida* expresses different types of LPS on its membrane, which allows further classification of the bacterium into 16 serotypes (1-16) based on somatic antigens. LPS is a major component of the gram negative cell envelope serving both structural and functional roles. The gram-negative outer membrane is an asymmetric lipid bilayer with scattered integral protein molecules. The inner membrane consists of glycerol-phospholipids, mainly phosphatidylethanolamine, and smaller amounts of phosphatidylglycerol and cardiolipin (Yethon and Whitfield, 2001). In contrast, the lipid portion of the outer membrane is composed almost entirely of LPS molecules. LPS molecules are amphiphilic glycolipids with unique structural features that allow for the selective permeability and barrier function of the outer membrane (Yethon and Whitfield, 2001).

In general, the LPS molecule consists of three regions: lipid A which is the hydrophobic part of the molecule that anchors LPS to the outer membrane, the core oligosaccharide which is a short chain of sugar residues with multiple phosphoryl substituents, and the O antigen which is a structurally diverse, serospecific polymer with oligosaccharide repeats. Lipid A is the most conserved part of the LPS molecule and it accounts for the low fluidity of the structure for this molecule. The oligosaccharide portion in the core of lipid A represents the integral part of the membrane, forming a tight barrier for charged ions and substrates. The negatively charged core oligosaccharides

bind calcium and magnesium to form a stable crosslink network between the LPS molecules contributing significantly to the outer membrane stability and integrity (Nikaido, 1995). Highly lipophilic, polycationic and ion-chelating substances can lead to disruption of the LPS integrity, damaging the membrane (Nikaido, 1995). In addition, minor reductions of Lipid A content (<30%) have been shown to render an *Escherichia coli* strain hypersensitive to antibiotics such as erythromycin and rifampin to which it was originally resistant (Vuorio and Vaara, 1992). Other properties of Lipid A include a series of biological responses observed in mammals following gram-negative sepsis. The term "endotoxin" has been applied to Lipid A to symbolize these biological responses. Lipid A is responsible for induction of endotoxic shock, fever, and non-specific activation of host lymphocytes and macrophages (Beutler and Rietschel, 2003). Severe endotoxemia plays a major role in the pathogenesis of hemorrhagic septicemia and has been associated with the development of clinical disease and the onset of the first clinical signs during experimental *P. multocida* B:2 infections (Horadagoda *et al.*, 2002).

Following infection and multiplication or death of gram-negative organisms, LPS is released in a free form or complexed with bacterial surface proteins (Heumann and Roger, 2002). The release of bacterial components in the host, locally or systemically, activates cells such as monocytes/macrophages, neutrophils and endothelial cells to generate an inflammatory response. Once LPS is released in the serum, it forms a complex with LPS-binding protein (LBP). The resulting LPS-LBP complexes bind the CD14 receptor, which is found on the membrane of monocytic cells (mCD14) or in soluble form in blood or fluids (sCD14). The bovine CD14 receptor has been cloned and has been demonstrated on alveolar macrophages (Yang *et al.*, 1995; Ikeda *et al.*, 1997).

Following activation of the cell (macrophages) by LPS, inflammatory cytokines such as TNFα, IFN-γ and IL-1 are produced which, when acting together, alter coagulation, induce fever and cause hypotension and tissue injury. They also trigger the release of secondary cytokines such as IL-6 and many chemokines which enhance sepsis. Experimental administration of LPS from *P. multocida* to buffaloes has been shown to induce the expression of the cytokines TNFα, IL-1 and IL-6 and the subsequent development of clinical signs such as fever and depression and physiological disturbances such as leukopenia followed by leukocytosis (Horadagoda *et al.*, 2002).

Heat shock proteins

Also known as stress proteins, heat shock proteins are induced when the cell is subjected to different types of environmental stress like heat, cold or oxygen deprivation. One of their functions as chaperons is to make sure that newly synthesized cell proteins are folded into the right shape and in the right place under these conditions. *P. multocida* expresses several heat-shock proteins *in vitro* when grown at 42°C but not at 32°C, and antibodies against four of these proteins (70, 60, 40 and 35 kDa) have been demonstrated in serum following infection (Love and Hirsh, 1994). The 60 kDa protein has been shown to be encoded by the *groESL* operon of an avian strain of *P. multocida* A:3 (Love *et al.*, 1995). This 60 kDa *P. multocida* protein is related to heat shock proteins of several other bacteria such as *Haemophillus ducreyi* (80% identity). Heat shock proteins thus provide protection to the bacterium from stressful conditions encountered within the host.

Toxin

Some strains of *P. multocida* express an extremely potent mitogenic substance known as dermatonecrotic toxin or *P. multocida* toxin (PMT). PMT is a major virulence factor of *P. multocida* serogroup D, which has been associated with progressive atrophic rhinitis in domestic and wild pigs (Dominick and Rimler, 1988). This disease is characterized by a marked localized osteolysis and osteoclastic resorption induced by PMT in the nasal turbinates that results in irregular growth of the snout, chronic respiratory disease, and poor weight gain (Hoffman, 1999).

The molecular mechanisms of action of this toxin are not well-understood; however, the toxin does act intracellularly on GTP-binding protein (Seo *et al.*, 2000). PMT stimulates several signaling cascades mediated by protooncogenes including those linked to phospholipase C, proteinase kinase C, and calcium mobilization (Lax and Thomas, 2002). Interestingly, the effects of PMT on tissue culture cells results in activation of the Rho-A signal transduction pathway and, consequently, the focal adhesion kinase (FAK) and Src family kinases. High levels of activity of these proteins have often been associated with various cancers and lead to cell transformation (Lax and Thomas, 2002). To date, bovine *P. multocida* isolates (A:3) have not been shown to express PMT.

Outer membrane proteins

Outer membrane proteins (OMPs) usually include several large, integral transmembrane proteins that traverse the lipid barrier, and several lipoproteins that are anchored in the lipid layer of the outer leaflet (Nikaido, 1988). These proteins have essential functions in membrane stability and the flow of substrates across the membrane (e.g. porins, which act as water-filled membrane pores). In addition, OMPs of Gram-

negative bacteria promote resistance to complement-mediated killing either by preventing the activation of complement pathways and/or blocking the formation of a lethal membrane attack complex on the bacterial membrane (Lin *et al.*, 2002).

P. multocida expresses several surface-exposed outer membrane proteins *in vivo* that may be important virulence factors. How these proteins contribute to virulence has not been determined in most cases; however, they appear to be important immunogens and are therefore worth mentioning. Lu *et al.,* (1988) showed that rabbit immune sera recognized 5 major OMPs of *P. multocida* A:3, having molecular masses of 27, 37.5, 49.5, 58.7 and 64.4 kDa. Similarly, Pati *et al.* (1996), found that *P. multocida* B:2 expresses several OMPs ranging from 88 to 25 kDa. Three of these OMPs, 44, 37 and 30 kDa, appeared to be immunodominant. Furthermore, Dabo *et al.*, (1997) demonstrated that bovine convalescent sera recognized several immunogenic, surface exposed, conserved OMPs in different *P. multocida* A isolates ranging from 18 to 68 kDa. Two major OMPs, a 35 and a 46 kDa protein, showed heat-modifiability and shared sequence homology with the *E. coli* OmpA and *H. influenzae* P1 proteins.

One of the most important OMPs of *P. multocida* is OmpH, a 37.5 kDa protein with porin activity (Luo *et al.*, 1997). Likewise, Gatto *et al.*, (2002) characterized another major OMP of *P. multocida* A:3 (Omp28) which is antigenic, surface exposed, and a member of the OmpA-porin family. Moreover, Kasten *et al.* (1995), showed that *P. multocida* expresses a 16 kDa protein with extensive sequence homology to the gene encoding the P6 protein of *H. influenzae*. This lipoprotein is thought to have a structural function within the bacterial cell. Similarly, *P. multocida* expresses an 87 kDa protein (Oma87) that shares significant homology with D15, a protective antigen of *H. influenzae*

(Ruffolo and Adler, 1996). In addition, *P. multocida* also expresses a 50 kDa OMP that has antiphagocytic activity (Truscott and Hirsh, 1988).

Iron-regulated outer membrane proteins

Genes encoding proteins homologous to proteins of other bacteria known to be involved in iron uptake or acquisition constitute over 2.5% of *P. multocida* genome (May *et al.*, 2001). May *et al.* (2001), used microarrays to determine the effects of iron-restricted conditions on the growth of *P. multocida* over time. They identified 174 genes whose transcription was altered in the absence of free iron. *P. multocida* expresses several IROMPs when grown *in vitro* under iron-restricted conditions or *in vivo*. The molecular weight of these proteins ranges from 35 to 109 kDa depending on *P. multocida* strain, culture media and/or iron chelator used (Ikeda and Hirsh, 1988; Snipes *et al.*, 1988; Glisson *et al.*, 1993; Veken *et al.*, 1994; Zhao *et al.*, 1995; Ruffolo *et al.*, 1998; Borkowska-Opacka and Kedrak, 2002). For example, Borkowska-Opacka and Kedrak (2002) found that bovine isolates type B:2 and A:3 expressed IROMPs ranging from 22 to 86 kDa and 20 to 104 kDa, respectively (Borkowska-Opacka and Kedrak, 2002). Similarly, we have observed that *P. multocida* A:3 strain 232 expresses four major high molecular weight IROMPs of 48, 80, 96 and 107 kDa (Confer, Prado unpublished observations). In addition, Zhao *et al.* (1995), showed that *P. multocida* A:5, a porcine isolate, expressed IROMPs of 74, 94, 99 and 109 kDa while the strain B80 expressed IROMPs of 74, 82, 94 and 99 kDa.

Iron-acquisition mechanisms of pathogenic bacteria and *P. multocida*

P. multocida, like other living organisms, requires iron for growth. Under aerobic conditions iron is found in its insoluble form Fe^{3+} at a concentration of 10^{-9} M, which is

100 times less than what is needed to support bacterial growth (Fe^{3+} concentration range of 10^{-7} M) (Ratledge and Dover, 2000). Furthermore, the availability of iron in the host is limited because it is bound intracellularly to ferritin and heme. Extracellular iron in serum or on mucosal surfaces is similarly bound to high affinity iron-binding glycoproteins such as transferrin and lactoferrin. During infection, inflammation or stressful conditions, host ferritin decreases the availability of iron even more by reducing the amount bound to the transferrin pool. To overcome this problem, bacteria have evolved iron-uptake systems consisting of sophisticated receptor proteins for recognition and transport of catalyzed iron (Ratledge and Dover, 2000). Thus, these proteins may be considered virulence factors as they are expressed *in vivo* and likely aid the bacterium in the acquisition of iron when faced with iron-restricted conditions (Veken *et al.*, 1994; Zhao *et al.*, 1995).

Siderophores

Under iron deficient conditions bacteria synthesize and secrete iron-chelating molecules, termed siderophores (low-molecular weight chemical iron chelators) (Lankford, 1973). At least one siderophore-like factor (multocidin) has been described for *P. multocida* (Hu *et al.*, 1986). Following synthesis, these molecules are secreted into the extracellular compartment where they can competitively capture Fe^{3+} ions from host iron-binding proteins or iron-binding compounds. The affinity or binding constants of siderophores is extremely high and varies between 10^{-20} and 10^{-52} M (Earhart, 1995). The ferric-siderophore complex then binds to a receptor located on the bacterial cell outer membrane where iron is removed and internalized. Subsequently, the iron complex is translocated through the outer membrane into the periplasmic space. This is an energy-

dependent step that is mediated by a complex of proteins designated TonB, ExbB and ExbD that are located on the cytoplasmic membrane (Postle and Kadner, 2003). A conformational change in TonB occurs in complex with its accessory proteins ExbB and ExbD following iron binding (Hancock and Braun, 1976; Higgs *et al.*, 2002; Postle and Kadner, 2003). The accessory proteins ExbB/D are important for the energy transduction to occur and most likely use the proton-motive force from the cytoplasmic membrane to convert TonB to an energized state (Postle and Kadner, 2003). The energy generated during this process is then transduced to the outer membrane transporters by the TonB. Once in the periplasm, the iron-siderophore complexes become a substrate for specific binding proteins. These are soluble proteins that capture the iron substrates and interact with cytoplasmic transport systems. The cytoplasmic membrane protein components belong to the family of ATP-binding cassette transporters (ABC transporters) and catalyze the final uptake of the iron-siderophore complex, an ATP-dependent event. The final step in the process is the release of Fe^{+2} by the iron siderophore. Some of the siderophores can be used again while others are degraded (Earhart, 1995).

Receptors for host-iron binding proteins

Another type of bacterial iron-uptake system involves the expression of high-affinity outer membrane receptors that bind directly to host iron-binding proteins such as transferrin, lactoferrin or hemoproteins (Ratledge and Dover, 2000). These receptors have several features in common. First, they are expressed as large trans-membrane proteins ranging from 80 to 120 kDa. Second, they have a conserved region in their N-terminal end known as the TonB-box. Third, they interact with TonB, a periplasmic protein. Fourth, they are expressed under iron-restricted conditions. Bacterial

transferrin- and lactoferrin-binding proteins have been characterized mainly in pathogens from the *Pasteurellaceae* and *Neisseriaceae* families (Schryvers and Gray-Owen, 1992; Schryvers and Stojiljkovic, 1999; Genco and Dixon, 2001; Ogunnariwo and Schryvers, 2001). These receptors are typically formed by two subunits designated transferrin-binding proteins A or B (TbpA and TbpB) and lactoferrin-binding proteins (LbpA and LbpB) (Ratledge and Dover, 2000). In *H. influenzae* for example, human transferrin has been shown to be a suitable substrate and is specifically recognized by two outer membrane receptor proteins encoded by *tbpAB* gene (Gray-Owen *et al.*, 1995). Conversely, bovine *P. multocida* isolates serogroups A and B:2,5 express a single novel transferrin receptor (TbpA) that efficiently mediates iron acquisition from bovine transferrin without the involvement of a second receptor protein (TbpB) (Veken *et al.*, 1994; Ogunnariwo and Schryvers, 2001).

The ability to use heme as a source of iron is widely distributed among bacterial pathogens. Two types of hemoglobin-binding proteins have been described (Schryvers and Stojiljkovic, 1999; Ratledge and Dover, 2000). The first and most common one involves the direct binding and processing of heme or hemoprotein by a TonB-dependent outer membrane receptor and subsequent passage of the released heme to the periplasmic space for transport into the cytoplasm (Ratledge and Dover, 2000). The second system requires the production of a soluble protein or hemophore, which interacts with the hemoproteins and returns heme to the cell surface, where it interacts with a TonB-dependent receptor (Ratledge and Dover, 2000). *H. influenzae* expresses specific hemopexin- and hemoglobin- or haptoglobin-binding protein complexes that allow it to utilize free hemin as well as protein bound hemin (Hanson *et al.*, 1992a; Hanson *et al.*,

1992b; Cope *et al.*, 1995; Wong *et al.*, 1995; Jin *et al.*, 1996; Maciver *et al.*, 1996; Jin *et al.*, 1999; Morton *et al.*, 1999). In contrast, *Serratia marcescens* produces an extracellular protein or hemophore (HasA) under iron-restricted conditions that is necessary for hemoglobin to be used as an iron source (Ghigo *et al.*, 1997; Letoffe *et al.*, 1999). The function of this protein is to remove heme from hemoglobin and to interact with HasR, a TonB-dependent outer membrane receptor (Ratledge and Dover, 2000). In the case of *P. multocida,* the iron-uptake systems involving heme have not been well characterized. However, based on the *P. multocida* genome sequence, several genes encoding potential TonB-dependent receptor proteins have been identified. Recently, Bosch *et al.* (2002b), demonstrated that a porcine strain of *P. multocida* serogroup D expresses a hemoglobin-binding protein encoded by the *hgbA* gene that is essential, iron-regulated, and highly conserved. Furthermore, Cox *et al.* (2003), showed that *P. multocida* A:1 expresses a hemoglobin-binding protein encoded by the gene *hgbB*.

Regulation of iron-uptake systems

Iron-dependent regulation of gene expression has been investigated in detail, showing that there is a hierarchy of gene expression control. The main regulatory protein found in many bacterial species is termed Fur (Fe uptake regulation) (Ernst *et al.*, 1978). It has been shown that the *fur* gene product encodes a repressor protein that binds Fe^{2+} and prevents gene transcription (Hantke, 1984). Therefore, Fur acts as a sensor for intracellular Fe^{2+} concentration. When the concentration of intracellular Fe^{2+} is low, the repressor function of Fur is lost and it can no longer interact with its binding sites that are located in the promoter region of iron regulated genes (Crosa, 1997). As a result, the expression of Fur-controlled genes is induced.

In *E. coli,* there is a *fur* mutant that expresses iron-induced gene products constitutively (Hantke, 1981). This mutant is viable, although it had impaired growth and there was evidence of oxidative DNA damage. Using this mutant, it was shown that impaired iron regulation leads to an increased sensitivity to hydrogen peroxide as well as spontaneous mutagenesis (Hantke, 1981). A *Staphylococcus aureus fur* mutant showed decreased virulence in a murine skin-abscess model (Bosch *et al.*, 2001). Interestingly, attempts to obtain *fur* mutants in *Pseudomonas aeruginosa, Neisseria gonorrhoeae, Neisseria meningitides* and *Vibrio anguillarum* were all unsuccessful, strongly suggesting that Fur plays an essential role in the viability of these bacteria (Touati, 2000). In addition, Fur regulation of cytoplasmic superoxide dismutase (SOD) has been observed in some bacteria (Touati, 2000). Thus, the deregulation of the control of iron acquisition in the presence of iron-sufficient conditions is conducive to oxidative stress in an aerobic environment (Touati, 2000). Superoxide favors the Fenton reaction:

$$(H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-})$$

by reducing free Fe^{2+}, leading to production of hydroxyl radicals that can damage the cell (Touati, 2000).

P. multocida also expresses a Fur protein (Bosch *et al.*, 2001; May *et al.*, 2001; Bosch *et al.*, 2002a). The *fur* gene for *P. multocida* has been cloned and found to encode a protein of 147 amino acids, sharing 89% identity with *H. influenzae* Fur and being negatively regulated by its own product (Bosch *et al.*, 2001). However, few experiments have addressed the function of Fur in *P. multocida.* Recently, Bosch *et al.*, (2001) demonstrated that the *ompH* gene is negatively regulated by the Fur protein. In addition, a *P. multocida fur* mutant was constructed and found to retain its virulence in a mouse

virulence assay (Bosch *et al.*, 2001). These results suggest that *P. multocida* is not negatively affected by high iron concentrations during infection and agree with previous findings demonstrating virulence of a *Salmonella* serotype Typhimurium *fur* mutant when inoculated intraperitoneally but not orally in a mouse-virulence model (Garcia-del Portillo *et al.*, 1993).

Immunological relevance of *Pasteurella multocida* virulence factors

Adhesins

As mentioned above, *P. multocida* genome encodes the gene for a filamentous hemagglutinin designated *pfhB* that has significant homology to *fhaB* of *B. pertussis*. FhaB is a major virulence factor in *B. pertussis*, mediating adherence to host cells (Adler *et al.*, 1999). It is also a major component of the acellular vaccines used to protect humans. Therefore, it is reasonable to think that antibodies against PfhB may prevent attachment and colonization of *P. multocida* in the host, thus providing resistance to the development of pneumonia.

P. multocida also expresses type 4 pili (Ptf4) that are similar to pili from other medically significant gram-negative pathogens. These proteins constitute the basis for vaccines against bovine keratoconjunctivitis and ovine foot-rot caused by *M. bovis* and *D. nodosus,* respectively (Adler *et al.*, 1999). In fact, antibodies raised against PtfA reacted with the 18 kDa protein from all *P. multocida* serogroup A strains as well as the serogroups B and D strains, a recombinant *M. bovis* β pilin, and the native *B. nodosus* fimbriae (Ruffolo *et al.*, 1997). These data demonstrate that PtfA is highly conserved among *P. multocida* strains. Therefore, it is also reasonable to speculate that antibodies

against PfhA/B and Ptf4 might prevent adherence of *P. multocida* to the host mucosal epithelium and thus development of pneumonia.

Sialidases

These enzymes are produced by bacterial pathogens including *P. multocida* and one of their functions is to cleave carbohydrate residues from host mucin allowing the bacterium to gain access to the cell surface. Sialidases are considered virulence factors and therefore, could be considered potential immunogens. Ifeanyi and Bailie (1992) demonstrated that mice receiving rabbit antiserum to *P. multocida* A:3 neuraminidase were protected against challenge. Furthermore, Straus *et al.* (1996a), showed that antiserum from rabbits immunized with purified *P. multocida* A:3 neuraminidase was able to reduce the enzyme activity by 40.3%, and cross-reacted with neuraminidases produced by other serotypes reducing their activity between 30 to 60%.

Polysaccharide Capsule

The protective capacity of capsular material purified from *P. multocida* isolates has been investigated to a certain extent. In general, polysaccharide capsules are poor immunogens (Penn and Nagy, 1976; Frank, 1989). One study by Penn and Nagy (Penn and Nagy, 1976) suggested that immunization of cattle with purified *P. multocida* capsular polysaccharide plus aluminium hydroxide as an adjuvant was protective; however, it was later demonstrated that these results might have been influenced by LPS contamination (Penn and Nagy, 1976; Muniandy *et al.*, 1992). In addition, vaccination of rabbits or mice with purified capsular extract was not protective (Penn and Nagy, 1976; Muniandy *et al.*, 1992).

Lipopolysaccharide

Purified *P. multocida* LPS is highly antigenic and appears to be a major immunogen in birds; however, this has not been the case for cattle, rabbits or mice (Confer, 1993; Adler *et al.*, 1996). Furthermore, anti-LPS monoclonal antibodies (mAb) were shown to be opsonic but not bactericidal in the presence of complement (Adler *et al.*, 1996).

Outer membrane proteins

Antibody responses against OMPs from different Gram-negative bacteria have been shown to enhance phagocytic and complement activity, as well as to inhibit bacterial adhesion to host cells (Wannemuehler and Galvin, 1994; Srivastava, 1998b). Likewise, it was shown that antibody responses to different *P. multocida* OMPs might be important for stimulating immunity and preventing the development of *P. multocida* pneumonia (Confer *et al.*, 1996). In fact, cattle vaccinated with live *P. multocida* develop antibodies to several OMPs that correlate with resistance to challenge (Confer *et al.*, 1996). Previously, it was shown that antibodies against OMP but not LPS protect rabbits against pasteurellosis (Lu *et al.*, 1991a). Vaccination of mice, chickens, and rabbits with *P. multocida* OMPs has resulted consistently in significant protection against challenge (Lu *et al.*, 1991b; Pati *et al.*, 1996; Ruffolo and Adler, 1996; Luo *et al.*, 1997). *P. multocida* A:3 strain 232 expresses a major heat-modifiable OMP (Omp28) that has been shown to be highly antigenic and able to induce a significant immune response following vaccination of mice. However, these antibodies did not protect mice from homologous challenge (Gatto *et al.*, 2002).

Iron-regulated outer membrane proteins

It has been hypothesized that OMPs that are expressed *in vivo* under iron-restricted conditions might be involved in cross-protective immunity, i.e., immunity protective against various *P. multocida* serotypes and serogroups. Glisson *et al.* (1993), showed that vaccination of chickens and turkeys with bacterins of *P. multocida* serotypes 1 or 3 grown in iron-restricted conditions stimulated cross-immunity but provided inconsistent protection. In contrast, Kennett *et al.* (1993), showed that mice vaccinated with either *P. multocida* 6:B whole cells expressing IROMPs or its OMP fraction had enhanced protection against challenge as compared to mice vaccinated with preparations not expressing IROMPs. Similarly, Ruffolo *et al.* (1998), demonstrated that mice vaccinated with outer membrane preparations of *P. multocida* type A:1 or A:3 expressing IROMPs were protected from homologous challenge but were only partially protected against heterologous challenge. In addition, Srivastava (1998a), showed that mice, rabbits and cattle immunized with *P. multocida* expressing IROMPs developed higher antibody responses and greater resistance to challenge than animals vaccinated with *P. multocida* not expressing IROMPs. Furthermore, rabbits vaccinated intranasally with *P. multocida* A:3 outer membranes containing IROMPs developed increased serum and mucosal antibodies, and exhibited more rapid intranasal clearance of *P. multocida* following challenge as compared to rabbits vaccinated with outer membranes devoid of IROMPs (Confer *et al.*, 2001). Vaccination with a recombinant *P. multocida* IROMP (HgbB) did not protect mice against homologous or heterologous challenge and insertional inactivation of *hgbB* did not affect the ability of *P. multocida* to bind hemoglobin or its ability to produce disease in a mouse model (Cox *et al.*, 2003 168). Thus, vaccination

with IROMPs can induce protective immunity in certain experimental models; however, anti-HgbB alone does not account for the afforded protection.

Immunity to *P. multocida*

Naturally acquired antibodies to *P. multocida* in cattle have been documented (Collins, 1977; Gresham *et al.*, 1984; Sawada *et al.*, 1985). Due to the commensal nature of this bacterium, cattle may develop an antibody response without developing disease. Thus, most of the naturally acquired immunity results from sub-clinical infection and/or the continuous contact with the microorganism. It has also been shown that sera from convalescent animals react with various antigens of *P. multocida,* indicating that these proteins are expressed *in vivo* during infection (Confer *et al.*, 1996). In addition, the presence of circulating antibodies to *P. multocida* antigens in cattle correlated with immunity in experimental bovine pneumonia (Panciera *et al.*, 1984; Confer *et al.*, 1996).

Acquired resistance by passive transfer or by vaccination with protective *P. multocida* antigens is humorally-mediated (Collins, 1977). However, it has also been postulated that cell-mediated as well as humoral immunity might contribute to the protection of vaccinated calves against *P. multocida* (Verma and Jaiswal, 1997). The protective capacity of antibodies can be passively transmitted to naïve animals with serum of vaccinated or convalescent animals (Woolcock and Collins, 1976). However, little is known about the character of the protective antibodies: their kinetics following vaccination, the antigen specificity of the antibody response, or the functional subclasses involved (Verma and Jaiswal, 1998).

Several methods have been used to quantitatively and/or qualitatively evaluate the humoral immune response of cattle to *P. multocida.* These are ELISA, immunoblots, and

densitometry among others (Confer *et al.*, 1996; Confer *et al.*, 2001; Gatto *et al.*, 2002). ELISA has been used to determine the levels of antibodies in vaccinated or convalescent animals. Western immunoblotting and densitometry have been used to identify individual bacterial proteins that are recognized by these antibodies and to quantify the level of the immune response to these proteins (Mosier *et al.*, 1989b; Confer *et al.*, 1996).

The protective capacity of the antibody response elicited in *P. multocida*-vaccinated animals has been tested using experimental challenge models. Results from such studies have varied depending on the model used. Models used have differed from each other in species (mice, cattle), age (young calves versus yearlings), sample size, pre-infection with bovine herpesvirus 1 (BHV-1 or IBR) or BVD, dose of inoculum, route of administration [natural (aerosol, intranasal, endotracheal) or unnatural (intratracheal, endobronchial, transthoracic or agar emboli)], time of challenge, observation times, and lung lesion evaluation (Frank, 1989; Srinand *et al.*, 1995). In addition, the type of *P. multocida* vaccine (live, killed, with or without adjuvant), the dose, frequency and route of administration can also influence the outcome of such studies. For many years the USDA has used a standard mouse potency test to evaluate the protective capacity of test bacterins against a standard bacterin following vaccination and challenge (Confer, 1993). Ideally, the protective capacity of vaccinal antibodies against *P. multocida* should be evaluated in cattle under field conditions. To date, published studies addressing morbidity and/or survival under field conditions have only been conducted in cattle vaccinated against hemorrhagic septicemia.

Passive transfer in dairy and beef calves

Limited information is available regarding the passive transfer, serum levels, and duration of maternal antibodies against *P. multocida* in calves. Gresham et al. (1984), showed that in general, cow colostrum contained antibodies to *P. multocida,* and the presence of maternal antibodies correlated with higher serum antibodies during the first 8 hours in newborn calves that had ingested colostrum. Interestingly, a second peak was observed between days 5 and 14 after birth. It was postulated that this peak most likely represents an active antibody response to *P. multocida* following natural exposure (Gresham *et al.*, 1984). Results from that study suggested that the level of *P. multocida* antibodies acquired by passive transfer was not likely to provide protection in calves against the bacteria (Gresham *et al.*, 1984). Interestingly, maternal antibody levels were higher in dairy calves as compared to beef calves. These results are not surprising and could be explained by the fact that *P. multocida* has been implicated in causing respiratory disease more often in dairy than in beef cattle (Bryson, 1985; Baker *et al.*, 1986; Ames, 1997). However, the intensity of antibody response depends on the herd, their vaccination history, prevalence of the disease, and different stressors among other factors.

Vaccines

Historically, vaccination with live *P. multocida,* regardless of route of administration, has been associated with significant immune responses and enhanced resistance to pneumonia following natural exposure or experimental challenge (Panciera and Corstvet, 1984; Panciera *et al.*, 1984; Mathy *et al.*, 2002). However, use of live vaccines has been shown to be associated with undesirable side effects such as injection

site abscesses and vaccine-induced disease (Buckley and Gochenour, 1924; Mosier *et al.*, 1989a; Verma and Jaiswal, 1998). Attempts at attenuation of *P. multocida* by prolonged growth and/or animal passage to enhance the protective effect was not successful (Verma and Jaiswal, 1998). In addition, vaccination of cattle and buffaloes with lyophilized bacteria either intradermally or subcutaneously was protective but was also associated with local reactions and in some instances death (Myint *et al.*, 1987; Myint and Carter, 1989, 1990; Carter *et al.*, 1991). In contrast, chemical alteration of *P. multocida* using different concentrations of diamino acridine salts (acriflavin) did produce a strain that induced protection against challenge in cattle, swine and sheep (Kucera *et al.*, 1981).

Wei and Carter (1978) developed a *P. multocida* type B streptomycin-dependent mutant that was highly immunogenic in mice and rabbits. Intranasal administration of a live streptomycin-dependent *P. multocida* A:3 mutant but not A:12 in rabbits was protective against challenge (Lu and Pakes, 1981). Furthermore, vaccination of cattle with a *P. multocida* A:3 streptomycin-dependent mutant correlated with lower clinical scores, reduced death rates and increased profitability (Kadel *et al.*, 1985). Conversely, vaccination of young dairy calves with a commercial live *P. multocida* streptomycin-dependent product did not improve calf performance or health (Aubry *et al.*, 2001). In addition, evaluation of *P. multocida* bacterins under field or experimental conditions when administered alone (formalized or heat-inactivated) or in combination with one of several different adjuvants [such as aluminum hydroxide (ALH) Freund's complete (FCA) or incomplete (FIA) adjuvant or saponin] showed that these bacterins were ineffective in preventing the disease and were also associated with undesirable side effects (Mosier *et al.*, 1989a; Verma and Jaiswal, 1998). On the other hand, oil-

adjuvanted vaccines containing an emulsifying agent such as lanolin, mineral oil or lanolin/paraffin were reported to be protective under field conditions (Verma and Jaiswal, 1998). *P. multocida* vaccines containing oil-aluminum or sodium alginate appeared to be protective and associated with only minor adverse effects (Verma and Jaiswal, 1998). Recently, Tabatabaei *et al.* (2002), constructed *aroA* attenuated derivatives of two *P. multocida* B:2 strains by allelic exchange of the native *aroA* sequence with a genetically modified *aroA* sequence that was disrupted by insertion of a kanamycin-resistance cassette or with marker-free *aroA* sequences containing an internal deletion. Intranasal or intraperitoneal vaccination with these *aroA* mutant strains conferred protective immunity in a *P. multocida* challenge mouse model.

Additional attempts to develop an effective vaccine against *P. multocida* pneumonia have focused on the identification of bacterial components with an immunogenic potential for constituting subunit vaccines. Vaccination of cattle with potassium thiocyanate (KSCN) extracts of *P. multocida* in FIA or modified FIA induced significantly higher antibody responses and faster pulmonary bacterial clearance than in controls following aerosol challenge with *P. multocida* (Mukkur, 1978). In addition, these antibodies were agglutinating, hemagglutinating, bactericidal and homocytotropic. Furthermore, as described in the previous section, *P. multocida* expresses different virulence factors such as FHA, TFP, OMPs and IROMPs, all of which are considered potential immunogens based on the evidence presented above.

Summary and statement of research problem

Pasteurella multocida is a complex bacterium affecting the respiratory tract of a broad range of host species. In North American cattle, *P. multocida* is associated with

enzootic pneumonia in dairy calves and chronic suppurative bronchopneumonia of beef cattle. Specifics regarding bacterium-host interaction and protective immunity have yet to be elucidated. Furthermore, available vaccines only afford partial protection at best against *P. multocida*-induced pneumonia in vaccinated animals. Recent advances in molecular biology have allowed identification and understanding of different *P. multocida* virulence factors and the role that they might play in pathogenicity and in the development of protective immunity. Among the many potential virulence factors, IROMPs are of particular interest as potential immunogens because they are surface-exposed and expressed *in vivo,* and could therefore play an important role in stimulating protective immunity. The IROMPs of *P. multocida* have been incompletely characterized, and their role in stimulating an immune response in cattle remains to be determined.

This research was performed to gain a better understanding of the role of *P. multocida* IROMPs in stimulating a protective immune response in cattle. The research objectives were as follows:

1. Determine the onset and duration of naturally acquired antibodies against *P. multocida* in dairy and beef calves.

2. Determine if an iron-regulated outer membrane protein preparation of *P. multocida* A:3 is immunogenic in cattle

3. Evaluate the ability of IROMPs to enhance resistance against *P. multocida*-induced pneumonia in a bovine pneumonia model.

4. Correlate the antibody responses to individual IROMPs with resistance against *P. multocida*-induced pneumonia and further characterize these proteins utilizing molecular biological techniques.

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CHAPTER II

PASSIVE AND NATURAL ANTIBODY RESPONSES OF CALVES TO *Mannheimia haemolytica* AND *Pasteurella multocida* A:3 ANTIGENS

Abstract

The dynamics and duration of maternally-derived antibodies as well as the onset of acquired immunity against *Mannheimia haemolytica* and *Pasteurella multocida* in dairy and range-pastured beef calves were investigated. Four groups of cattle were used in this study [2 groups of dairy calves vaccinated with a commercial *M. haemolytica* culture supernatant and *P. multocida* antigen vaccine (Presponse-HM®) at 70 or 120 days, and 2 groups of beef calves that were unvaccinated]. Serum antibody responses were measured by enzyme-linked immuno-assay for antibodies of the IgG1, IgG2 and IgM isotypes binding *M. haemolytica* whole cells (WC) or leukotoxin (LKT) and *P. multocida* outer membrane proteins (OMPs). Comparisons of mean antibody responses to *M. haemolytica* LKT and WC and *P. multocida* OMPs were made within each group. Maternally-derived antibodies against *M. haemolytica* and *P. multocida* were short-lived and varied between calves from different production systems (average 30 days for dairy calves and 60-120 days for beef calves). Autogenous antibody production against *M. haemolytica* and *P. multocida* started between 60-90 days of age for all groups. Vaccination of dairy calves with Presponse-HM® was not associated with significant increases in antibody responses following administration of the vaccine. Based on the results of this study, vaccination of dairy or beef calves should be performed at 3 to 4 months of age.

Introduction

Pasteurella multocida A:3 and *M. haemolytica* are common inhabitants of the upper respiratory tract of healthy cattle and have been primarily associated with cases of enzootic pneumonia in dairy or housed calves and respiratory disease in feedlot animals, respectively (Ames, 1997). *P. multocida*-associated pneumonia in dairy calves continues to be a disease of great economical significance despite research efforts directed towards identifying potential risk factors or other etiological agents involved and the development of efficacious vaccines. The risk of pneumonia for dairy calves is the highest around 5 to 6 weeks of age (Virtala *et al.*, 1996). Some of the risk factors associated with the development of pneumonia include low post-colostrum IgG, type of housing for calves, and housing calves with adult cattle (Virtala *et al.*, 1999). One of the effects of pneumonia in the first 3 months of life is a decrease in total body weight (Virtala *et al.*, 1996).

Calves are naturally born hypogammaglobulinemic because of the syndesmochorial character of the ruminant placenta which prevents prepartum transfer of immunoglobulins from the cow (Barrington and Parish, 2001). During the first 24 hours of life, calves must ingest and absorb colostral immunoglobulins from their sero-positive dams in order to acquire passive immunity. Colostrum is rich in immunoglobulin G (IgG) of which 85% is IgG1 (Barrington and Parish, 2001). The half-life of maternally-derived antibodies in the calf is between 11.5 and 16 days (Barrington and Parish, 2001).

Antibodies to *M. haemolytica* and *P. multocida* have been found in colostrum of dairy cows and these are passively transferred to calves; however, little is known regarding their fate (Gresham *et al.*, 1984). Furthermore, while *M. haemolytica* and *P.*

multocida have been associated with cases of respiratory disease in beef calves, information regarding the passive transfer of anti-*M. haemolytica* or *P. multocida* antibodies or dynamics of *P. multocida* infection in range-pastured beef calves is limited.

It is known that passive immunity is an obstacle for induction of active immunization, and therefore, the duration of maternal antibodies must be taken into account when designing vaccination regimens (Morein *et al.*, 2002). Persistence of maternally acquired antibodies in the calf varies substantially, ranging from days to months. For instance, some anti-viral antibodies such as anti-bovine viral diarrhea (BVD) IgG are known to persist in the calf for up to 9 months following passive transfer (Tizard, 2000). The duration of maternal anti-*M. haemolytica* and anti-*P. multocida* antibodies is not known in beef calves, nor is the beginning of spontaneous antibody production to these bacteria as a result of natural infection. Among the different isotypes, IgG2 appears to be the most important antibody for protection against pyogenic extracellular infections; therefore, understanding the dynamics of anti-*M. haemolytica* and anti-*P. multocida* antibodies with respect to duration and immunoglobulin isotypes needs to be studied (Corbeil *et al.*, 1997).

The objectives of this study were to determine and compare the dynamics and duration of maternal antibodies to *P. multocida* and *M. haemolytica* in dairy and beef calves, and to determine the age at onset of acquired immunity to these agents.

Materials and methods

Animals

Calves from three different farms were used in this study (table 1). The first group consisted of 18 Holstein calves from the Oklahoma State University dairy farm born

during a 2-month period in the fall of 1998. Each calf was separated from its dam at birth and received pooled colostrum by bottle. Diet consisted of a commercial milk replacer twice a day and calf starter ration ad libitum. Housing consisted of individual hutches with unlimited access to water. Sera were collected on days 0, 2, 30 and then every 30 days for 6 months. Dairy calves were divided into two groups (V70, V120) based on vaccination with a commercial *M. haemolytica/P. multocida* product (Presponse®HM) at 70 or 120 days of age. Calves were also vaccinated against respiratory viruses (IBR, PI3 and BVD) and Leptospira at the same time (Kirkpatrick *et al.*, 2001). Two groups of beef calves were also used. Group B1 consisted of 9 Hereford calves, and Group B2 consisted of 14 Maine Anjou crossbred calves born during the fall of 2000. Calves from groups B1 and B2 were kept with their dams on range pasture from birth to weaning. Sera were collected after birth and, when weather conditions allowed, every 30 days until calves were weaned at 180 days of age.

Serum antibody responses to *M. haemolytica* and *P. multocida* antigens

Serum IgG1, IgG2 and IgM antibodies to *M. haemolytica* leukotoxin (LKT) and whole cells (WC), and *P. multocida* outer membrane proteins (OMPs) were determined by ELISA as previously described with minor modifications (Confer *et al.*, 1985; Confer *et al.*, 1996; Confer *et al.*, 1997; Hodgins and Shewen, 2000). The *P. multocida* A:3 and *M. haemolytica* A1 strains used for antigen preparation were originally isolated from pneumonic steers. LKT was prepared as previously described (Confer *et al.*, 1997). For obtaining WC, formalin-killed *M. haemolytica* was prepared from a washed 24-hour culture by suspending cells in 0.4% formalinized saline at a concentration determined spectrophotometrically to be 1.850 at O_{D650} (Confer *et al.*, 1997). *P. multocida* OMPs

were prepared by extraction with Sarkosyl as previously described (Squire *et al.*, 1984; Dabo *et al.*, 1997).

Wells of 96-well polystyrene EIA/RIA microtiter flat bottom high binding plates (COSTAR #9018 Corning Inc., Corning, NY) were coated with LKT at 50 ng per well, WC at an optical density reading equivalent to 10^8 colony forming units (CFU) of a 24-hour culture, or with 100 ng of *P. multocida* OMPs. Purified LKT and OMPs were diluted in 0.06 M carbonate buffer (pH 9.6). Primary antisera were diluted in block buffer solution consisting of 0.5% fish skin gelatin (Norland Products Inc, New Brunswick, New Jersey, USA) and 0.05% Tween-20 in PBS (pH 7.4) and assayed in triplicate. Bound antibodies were detected using mouse monoclonal antibody to bovine IgG1 at a 1:200 dilution or IgG2 at 1:100 dilution (Serotec, Station Approach, Kidlington, Oxfordshire, England). Monoclonal anti-antibody was detected using an alkaline phosphatase-conjugated goat anti-mouse IgG H+L reagent at 1:1000 (Caltag Laboratories, San Francisco, California, USA). Enzymatic activity was assayed using the BluePhos®Microwell Phosphatase Substrate System which contains soluble BCIP (5-bromo-4-chloro-3-indolyl-phosphate) (KPL). Optical densities of the wells were read at 650 nm. Antibody responses are expressed as nanograms of IgG binding compared to a standard immunoglobulin curve for each plate.

Serum antibodies of the IgM isotype binding *M. haemolytica* LKT and WC, and *P. multocida* OMPs were determined as described above but utilizing mouse monoclonal antibody to bovine IgM at a 1:2000 dilution (Sigma Chemical Co.). In order to minimize non-specific binding of IgM to the polystyrene plate, a few modifications were made to the protocol. A blocking step (2.0% fish skin gelatin in PBS, 200 µl/well for 1 hour at

37°C) was added after the plates were coated with antigen. Serum samples were diluted in a solution of 1.5% Tween-20 in PBS (with 0.29 M NaCl). In addition, for each serum sample, 3 control wells not coated with antigen were used to monitor background signal. Mean optical density due to non-specific binding was subtracted from the mean optical density observed with antigen. Antibody responses are expressed as nanograms of IgM binding using a set of immunoglobulin standards.

Statistical analysis

Comparisons of mean antibody responses to *M. haemolytica* leukotoxin and whole cells, and *P. multocida* OMPs were made within each group by PROC MIXED analysis with repeated measures (SAS System 8.02 TS Level 02M0) as well as comparisons between groups of vaccinated dairy calves (V70 or V120). Significant differences were noted when $P < 0.05$.

Results

Antibody responses to *M. haemolytica* Leukotoxin and Whole Cells

Dairy calves – As depicted in figs. 1 and 2, anti-LKT and anti-WC IgG1 concentrations were undetectable on day 0 in all calves. By day 2, anti-LKT and anti-WC IgG1 concentrations had significantly increased in both groups. This increase was followed by a gradual decline reaching baseline values by day 30, and IgG1 values remained at this level for the duration of the study. Interestingly, the V70 group had significantly higher anti-LKT IgG1 levels at day 2 than V120 group. Anti-LKT and WC IgG2 concentrations showed two significant increases during the sampling period; the first one occurring by the second day for both groups that was more subtle than the increase observed with anti-LKT and anti-WC IgG1 concentrations. The anti-WC IgG2

concentrations declined on day 30 for group V70. The second increase in anti-LKT and WC IgG2 concentrations took place between days 60 and 90 for both treatment groups that continued to increase through the duration of the sampling period. The V70 group developed significantly higher anti-LKT IgG2 levels than the V120 group at days 2 and 90. Similarly, anti-LKT and anti-WC IgM antibodies significantly increased by day 2 and decreased by day 30 for both treatment groups. However, anti-WC IgM concentrations significantly increased at days 90 and 120 for V70 and V120 groups respectively.

Beef calves – Anti-LKT IgG1 concentrations significantly decreased by day 60 for group B1 and by day 30 for group B2 and continued to decrease to baseline values, and remained throughout the duration of the study (figs. 3 and 4). No significant changes were observed in anti-WC IgG1 concentrations at any time during the sampling period for group B1; however, in group B2, anti-WC IgG1 concentrations had significantly decreased by day 30 and continued decreasing until the end of the experiment. Anti-LKT IgG2 concentrations significantly increased by day 155 in the B1 group and continued to increase thereafter. However, anti-LKT IgG2 concentrations significantly decreased by day 30 in the B2 group and continued to decrease mildly until day 120 where an increase was evident, but this was not statistically significant. In the case of anti-WC IgG2 antibodies there was a significant increase by day 140 in both groups that continued to increase until the end of the sampling period. Anti-LKT IgM antibodies gradually increased until becoming significant by day 155 in the B1 group and continued to increase thereafter. In the B2 group anti-LKT IgM antibodies significantly decreased by day 30 followed by mild fluctuations that were not significant by the end of the study.

Anti-WC IgM concentrations were observed to fluctuate throughout the duration of the study for group B1; however, these changes were not statistically significant. In the case of group B2, anti-WC IgM antibodies significantly increased by day 60 followed by mild increases that were not significant.

Antibody responses to *P. multocida* Outer Membrane Proteins

Dairy calves – Anti-OMP IgG1 antibodies significantly increased by day 2 and decreased by day 30 in both treatment groups followed by subtle changes thereafter (fig. 1 and 2). By the end of the study period anti-OMP IgG1 antibodies had significantly increased for V70 group. Anti-OMP IgG2 concentrations mildly increased by day 2 for group V70; however, significantly higher increases were observed by day 90 and thereafter for both groups. Similarly, anti-OMP IgM antibodies significantly increased by the second day and decreased by day 30 in both groups followed by a mild increase at day 60 for V70 and day 120 for V120.

Beef calves – There was a mild decrease in anti-OMP IgG1 antibodies during the first 90 days for group B1 but this was not statistically relevant and was followed by a significant increase that carried over to the end of the study period. Calves from the B2 group had a significant decrease in anti-OMP IgG1 antibodies by day 30 that continued to decrease by day 60 followed by a plateau. In the case of anti-OMP IgG2 there was a significant increase antibody concentration by day 140 for calves in group B1 and by day 120 for calves in group B2, which remained significantly elevated through the duration of the sampling period. Anti-OMP IgM antibody concentrations significantly increased by day 60 and decreased by day 90 for calves in group B1. There was a significant increase in these antibodies by day 140 which remained significantly increased through the

duration of the study for calves in group B1. In calves from group B2; however, there was a significant decrease in anti-OMP IgM by day 30 and followed by a significant increase on day 120, remaining significantly increased until the end of the sampling period.

Discussion

Results of the present study show that acquired immunity to *M. haemolytica* and *P. multocida* by passive transfer is short lived and varies among calves raised in different production systems. Variability in the intensity of the antibody response to different bacterial antigens tested seemed to be dependent on age and type of cattle. These results are in agreement with previous studies that showed that maternally acquired antibodies against *P. multocida* or *M. haemolytica* in Holstein calves waned between 2 to 4 weeks of age, whereas autogenous antibody production began shortly afterwards (Hodgins and Shewen, 1998; El-Eragi *et al.*, 2001).

The time that it took passively acquired IgG1 antibodies against *M. haemolytica* and *P. multocida* to reach the lowest point varied among farms, ranging from 30 days for dairy calves to 60-120 days for beef calves. The temporal persistence of maternally derived antibodies against *M. haemolytica* and *P. multocida* in calves is similar to the life-span of colostral antibodies against respiratory viruses (IBR, BVDV, PI-3V and BRSV), where passively-acquired antibodies last an average of 37 to 116 days (Kirkpatrick *et al.*, 2001; Munoz-Zanzi *et al.*, 2002). It is important to know the duration of passively acquired antibodies when designing a vaccination program as the presence of maternal antibodies might reduce or negate the effectiveness of the vaccine. Based on

the results of this study, the ideal time to perform vaccination against these agents would be when calves reach three to four months of age.

The length of time that maternally derived antibodies are measurable in a calf is directly proportional to the amount of immunoglobulins ingested in the first 24 hours of age. We observed a more rapid decline in IgG1 antibody concentrations in the dairy groups (30 days) than in the beef groups (60-90 days). The difference might be found in the type of production systems and management practices. It has been well documented that the keeping the calf with the dam correlates with higher absorption of Ig from the colostrum by the calf independent of the amount consumed. Dairy calves are separated from their dams at birth and this may have a detrimental effect on the amount of Ig acquired by passive transfer. Furthermore, dairy cows are bred for higher milk yields, which might result in diluted and therefore lower quality colostrum. In addition, vaccination history and age of the dam could potentially affect the quality of the colostrum.

In previous studies, Hodgins and Shewen (1998; 2000) showed that vaccination of colostrum fed or deprived calves at 2-4 weeks of age or 6-8 weeks of age with a *M. haemolytica* culture supernatant vaccine (Presponse®) was associated with serocoversion. The antibody responses in these studies were age-dependent, being higher in the older calves. In our study, vaccination of dairy calves with the same *M. haemolytica* culture supernatant vaccine containing *P. multocida* antigens at 70 or 120 days was not associated with significant increases in antibody responses. Instead, the increases in antibody concentrations that we observed starting around day 60 could be better explained as autogenous seroconversion due to natural exposure to these bacteria.

Most calves showed autogenous antibody production against *M. haemolytica* and *P. multocida* starting between 60 and 90 days of age as evidenced by anti-bacterial antibodies in IgG2. However, no clinical cases of respiratory disease were reported during this time. Spontaneous seroconversion is possible and is highly associated with colonization of the nasal passages with these pathogens as normal flora. Under stressful conditions, these pathogens most likely become more prevalent in the nasal cavity resulting in increased concentrations of bacteria in the inhaled air and consequently the development of an immune response. Grey and Thomson (1971) showed that increased colonization of the nasal passages by *M. haemolytica* was associated with the presence of this bacterium in tracheal air. In addition, environmental conditions during the winter months when samples were collected for this experiment were highly variable, ranging from mild weather to freezing rain and snow. Abrupt changes in weather can be stressful enough to allow for some bacteria to proliferate. These observations are in agreement with a previous study by Conlon *et al.* (1995), in which they concluded that the commensal nature of *M. haemolytica* in the upper respiratory tract of cattle stimulates a natural immune response in cattle. Subsequent vaccination results in an anamnestic response. In our study, the beef calves received no vaccination at all and autogenous antibody responses were evident between 60 to 90 days.

It has been shown that failure to obtain maternally-derived antibodies by passive transfer (low post-colostral total IgG) positively correlates with a higher risk for development of pneumonia in dairy heifers during the first 3 months of life (Virtala *et al.*, 1999). Our study demonstrated that martenally-derived antibodies against *M. haemolytica* and *P. multocida* decline quickly (average of 30 days), being present at low

concentrations during the time that dairy heifers are at their highest risk for pneumonia. However, when Virtala *et al.* (1996), evaluated specific titers to these agents and correlated them with post-colostral total IgG titers, no association was found. In contrast, maternally-derived antibodies against *M. haemolytica* and *P. multocida* in beef calves seemed to decline more slowly than in dairy calves, which might explain in part why pneumonia does not appear to be clinically significant in nursing beef calves while they are on range pasture.

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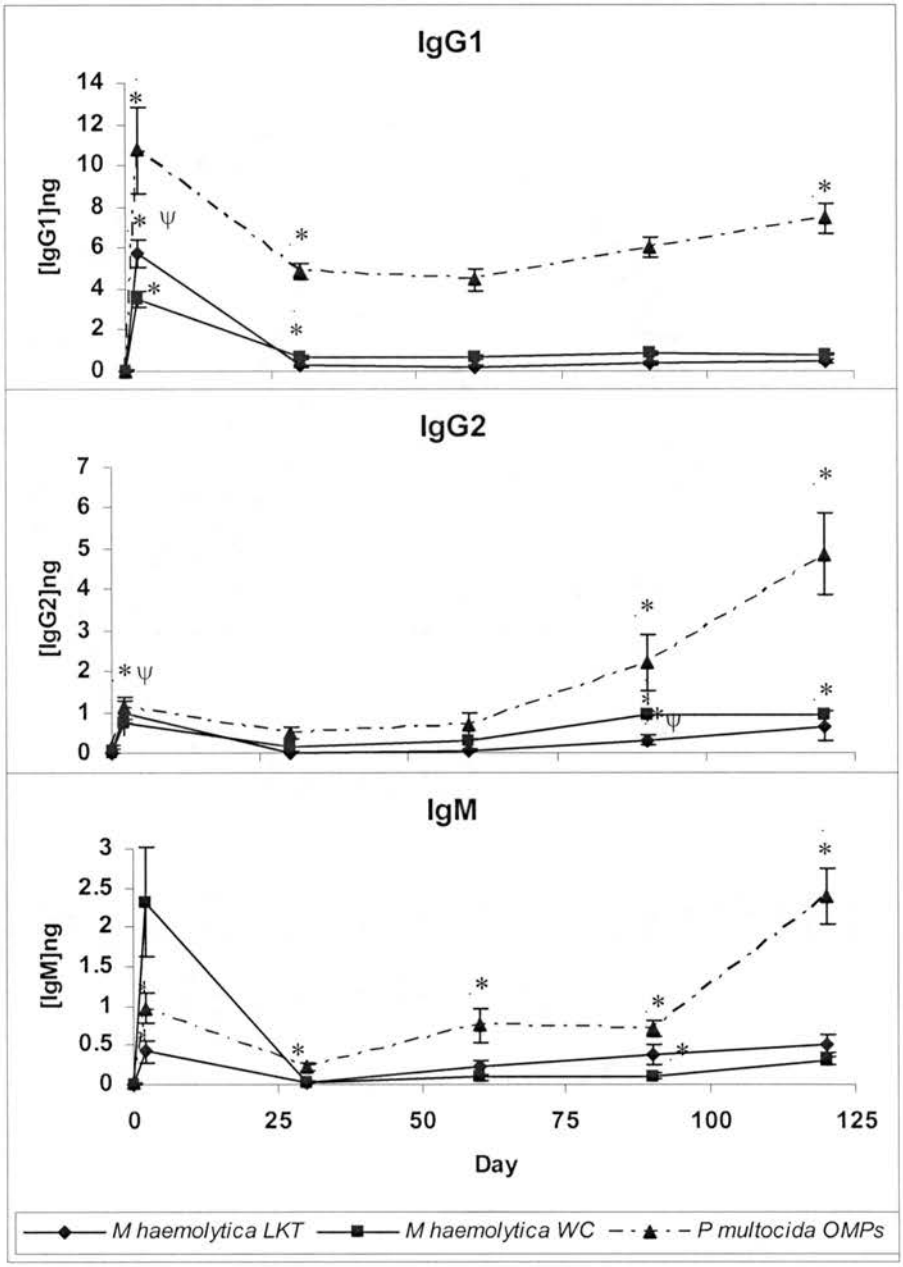
Table 1. Experimental design depicting the different groups of cattle used in this study.

Group	No. of Calves	Type of Cattle	Breed	Housing	Vaccination	Duration of Study
V70	9	Dairy	Holstein	Hutches	Presponse®HM Titanium5L5	120 days
V120	9	Dairy	Holstein	Hutches	Presponse®HM Titanium5L5	174 days
B1	9	Beef	Hereford	Pasture	None	240 days
B2	14	Beef	Maine-AnjouX	Pasture	None	180 days

Fig. 1. Serum antibody responses of dairy calves vaccinated with Presponse®HM at 70 days to *M. haemolytica* leukotoxin and whole cells, and *P. multocida* OMPs. Data are expressed as nanogram concentration of IgG1, IgG2 or IgM. Data shown are the means and standard error of the mean (SEM). A $P<0.05$ is considered significant. Differences (*) from control or (Ψ) between treatment groups are noted.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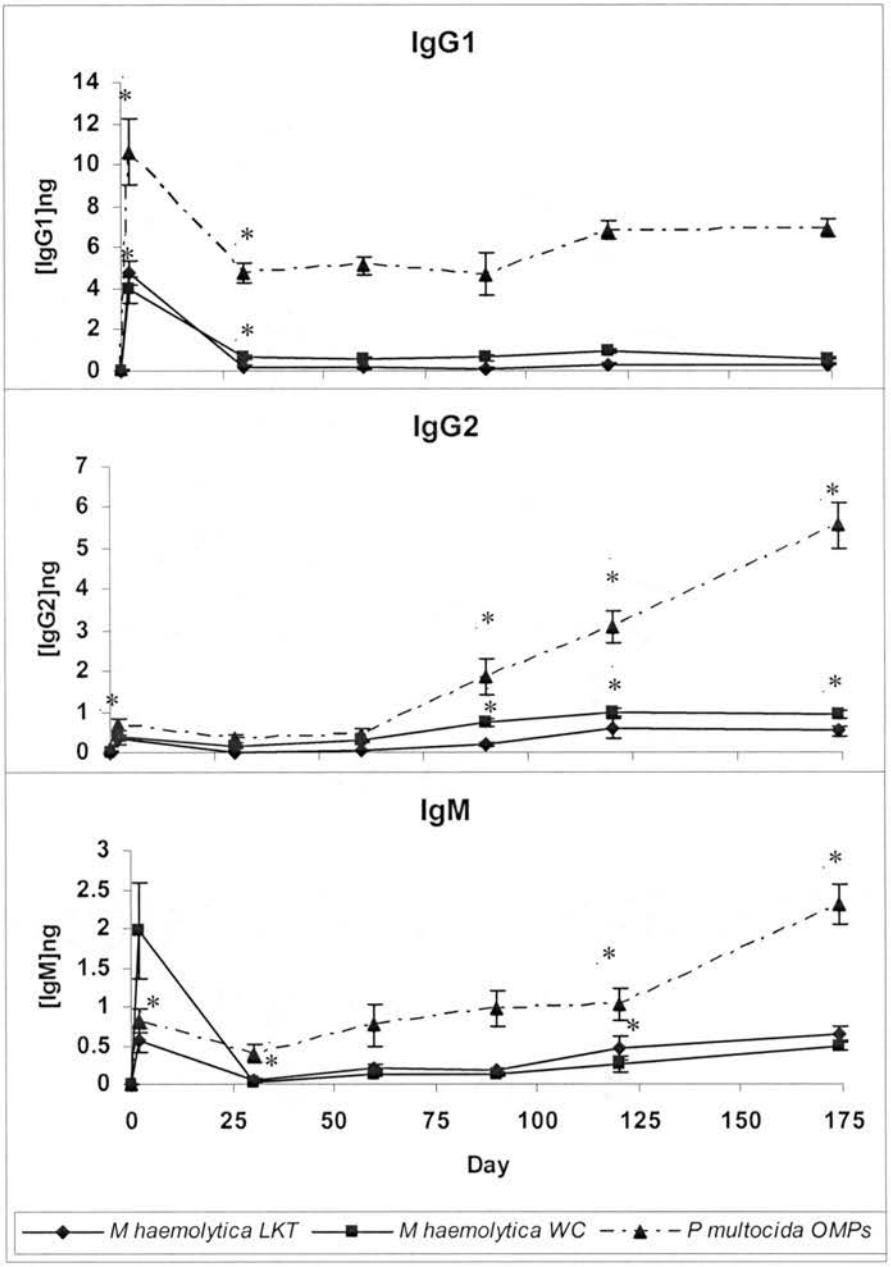
2
1
0
IgM
[IgM]ng
3
2.5
2
1.5
1
0.5
0
0
25
50
75
100
125
Day
M haemolytica LKT
M haemolytica WC
P multocida OMPs

Fig. 2. Serum antibody responses of dairy calves vaccinated with Presponse®HM at 120 days to *M. haemolytica* leukotoxin and whole cells, and *P. multocida* OMPs. Data are expressed as nanogram concentration of IgG1, IgG2 or IgM. Data shown are the means and standard error of the mean (SEM). A $P<0.05$ is considered significant (*).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0
IgM
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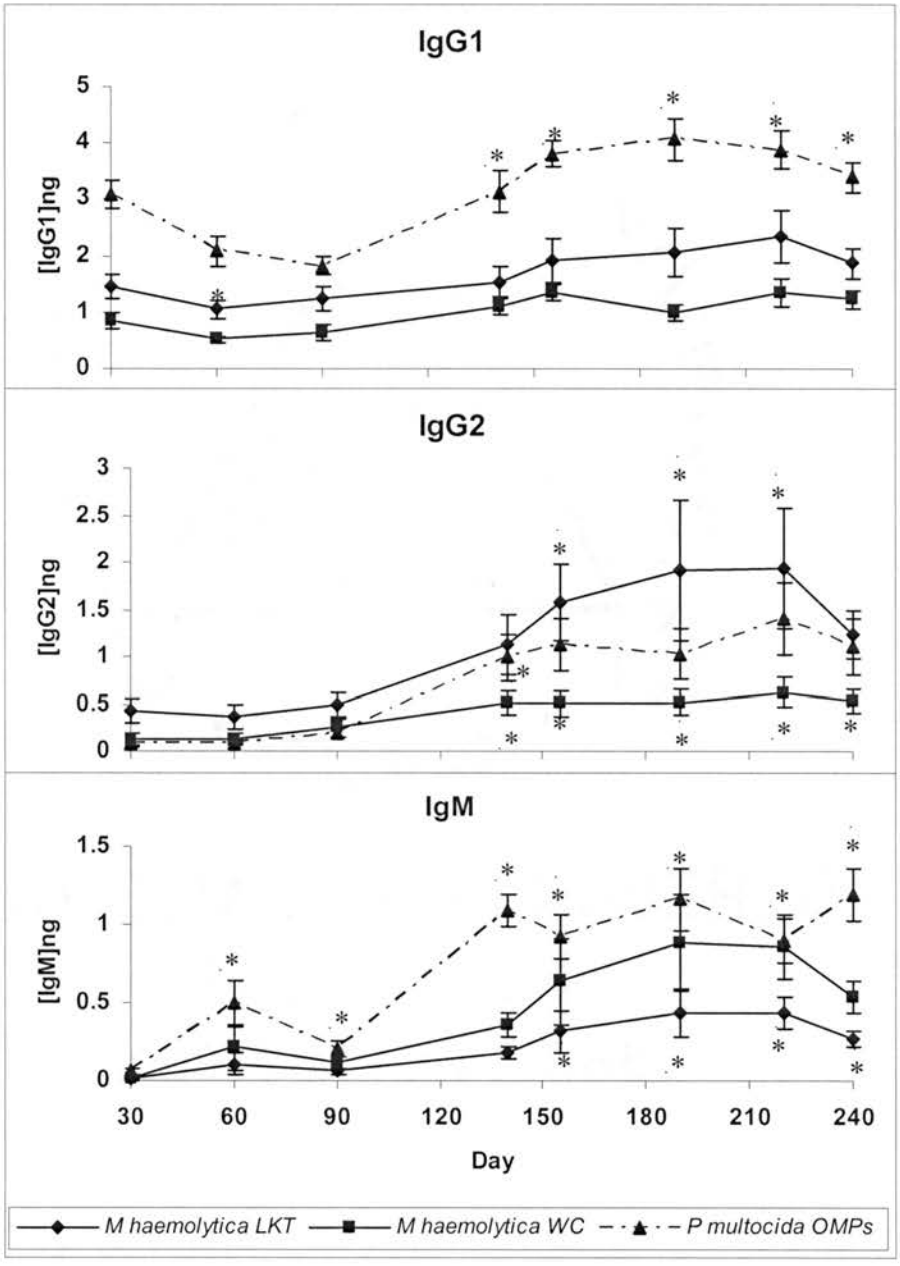
3
2.5
2
1.5
1
0.5
0
0
25
50
75
100
125
150
175
Day
M haemolytica LKT
M haemolytica WC
P multocida OMPs

Fig. 3. Serum antibody responses of beef calves (B1) to *M. haemolytica* leukotoxin and whole cells, and *P. multocida* OMPs. Data are expressed as nanogram concentration of IgG1, IgG2 or IgM. Data shown are the means and standard error of the mean (SEM). A $P<0.05$ is considered significant (*).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IgM
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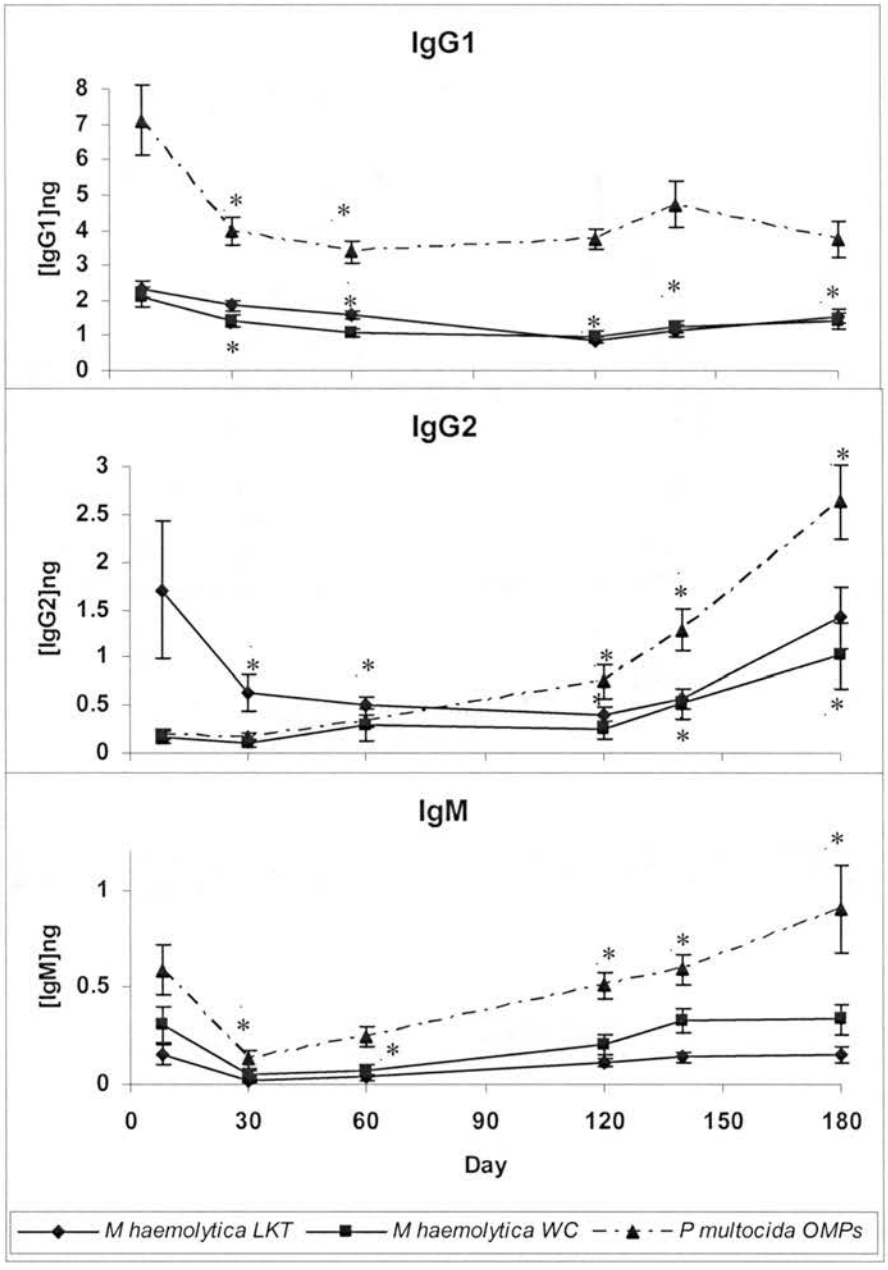
1
0.5
0
30
60
90
120
150
180
210
240
Day
M haemolytica LKT
M haemolytica WC
P multocida OMPs

Fig. 4. Serum antibody responses of beef calves (B2) to *M. haemolytica* leukotoxin and whole cells, and *P. multocida* OMPs. Data are expressed as nanogram concentration of IgG1, IgG2 or IgM. Data shown are the means and standard error of the mean (SEM). A $P<0.05$ is considered significant (*).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Day
M haemolytica LKT
M haemolytica WC
P multocida OMPs

CHAPTER III

VACCINATION OF CATTLE WITH IRON-REGULATED OUTER MEMBRANE PROTEINS OF *Pasteurella multocida* A:3 AND MOLECULAR CHARACTERIZATION OF HasR, A MAJOR IROMP

Abstract

The iron-regulated outer membrane proteins (IROMPs) of *Pasteurella multocida* A:3 (Pm232), a bovine isolate, were investigated as potential immunogens in cattle. We addressed the ability of *P. multocida* IROMP-enriched fractions to induce antibody responses in cattle by different vaccination strategies and the protective efficacy of these antibodies using a *P. multocida*-induced pneumonia challenge model. Vaccination of cattle with outer membrane-enriched fractions derived from *P. multocida* A:3 grown on either iron-depleted (IROMPs) or iron-sufficient (OMPs) conditions induced significant antibody responses that correlated with smaller lung lesion scores. Sarcosyl extractions of outer membranes from *P. multocida* grown under iron-depleted conditions were analyzed by using SDS-PAGE, western immunoblots and densitometry, revealing 4 major polypeptides in the IROMPs preparation that included a 96 kDa protein band. Further analysis of the 96-kDa protein performed by mass spectrometry generated peptide sequences with high level of homology to the heme acquisition system receptor (HasR) of avian *P. multocida* (strain Pm70). The *hasR* gene from *P. multocida* Pm232 was cloned sequenced, and the predicted protein characteristics determined. Our results indicate that antibody responses in cattle are induced effectively by *P. multocida* IROMPs, confirming the idea of IROMPs as potential immunogens and should be further considered in recombinant vaccine strategies.

Introduction

Pasteurella multocida is a common inhabitant of the respiratory tract of many species including cattle. In North America, *P. multocida* is associated with enzootic pneumonia in dairy calves and a subacute to chronic bronchopneumonia in beef cattle (Frank, 1989). The disease in yearling or feedlot cattle is part of the shipping fever complex. The interaction of multiple factors such as previous exposure to respiratory viruses or *Mycoplasma*, stressful management practices, and/or severe weather conditions predisposes cattle to developing bovine respiratory disease (BRD). In addition, respiratory disease (prevalence 28%) is the leading cause of death in production cattle, and the overall economic impact of BRD on the cattle industry has been estimated at 1 billion per year (Griffin, 1997; USDA *et al.*, 1997). In lieu of the economic significance of BRD, considerable efforts have been made in order to produce an efficacious vaccine. However, to date there are no biologicals that consistently and completely protect cattle from BRD.

P. multocida is a gram-negative coccobacillus that has been classified serologically into serogroups A, B, D, E and F based on its capsular antigen (CPS), and into serotypes 1-16 based on the nature of the lipopolysaccharide (LPS) (Carter, 1967; Heddleston *et al.*, 1972; Rimler and Rhoades, 1987). *P. multocida* strains most commonly isolated from cases of bovine pneumonia belong to serogroup A serotype 3 (Dyer, 1982; Frank, 1989; Dabo *et al.*, 1999). *P. multocida* expresses several antigens that have been identified as potential immunogens. These antigens include a CPS, LPS, adhesins such as type 4 pili (PtfA), outer membrane proteins (OMPs), and iron-regulated OMPs (IROMPs) (Confer, 1993; Ruffolo *et al.*, 1997). Yet, the immunity of the bovine host

against *P. multocida* still remains to be elucidated (Confer, 1993). Sera from cattle vaccinated with live *P. multocida* contain antibodies against bacterial antigens, suggesting *in vivo* expression of these polypeptides (Confer *et al.*, 1996; Dabo *et al.*, 1997). Therefore, these virulence factors must be important for the bacterium during infection and might play a role in stimulating protective immunity in cattle.

In general, all bacterial species rely on iron acquisition for growth and replication, and the outcome of bacterial infection depends to a certain extent on their ability to compete for iron (Bullen and Griffiths, 1999). The availability of free iron *in vivo* is limited due to the presence of high affinity iron-binding host proteins such as transferrin, lactoferrin, ferritin, serum albumin, hemopexin, hemoglobin, and other iron-storage proteins (Ratledge and Dover, 2000). Therefore, pathogenic bacteria such as *P. multocida* have evolved various strategies to scavenge iron from host sources. One such system consists of high-affinity low molecular weight iron chelators or siderophores, whereas another system involves the expression of receptors for host-iron binding proteins on the bacterial outer membrane (Ratledge and Dover, 2000).

Over 2.5% of the *P. multocida* genome constitutes genes encoding homologues to proteins of other bacteria that are known to be involved in iron uptake or acquisition (Confer *et al.*, 2001). Using DNA microarrays the transcriptional regulation of 174 genes of *P. multocida* were found to be controlled by iron-restricted conditions (May *et al.*, 2001). *P. multocida* expresses outer membrane proteins of different molecular weight *in vivo* or *in vitro* under iron-restricted conditions. The molecular weight of these IROMPs varies according to *P. multocida* strain, culture media and/or iron chelator used, and range from 35 to 109 kDa (Ikeda and Hirsh, 1988; Snipes *et al.*, 1988; Glisson *et al.*,

1993; Veken *et al.*, 1994; Zhao *et al.*, 1995; Ruffolo *et al.*, 1998; Borkowska-Opacka and Kedrak, 2002). These proteins likely mediate iron-acquisition *in vivo*, and thus their expression may be associated with virulence. Several IROMPs of *P. multocida* have been identified as receptors for specific host iron-binding proteins such as transferrin- (TbpA), lactoferrin- (Lbp), or hemoglobin-binding protein receptors (HgbA/B) (Veken *et al.*, 1994; Ratledge and Dover, 2000; Ogunnariwo and Schryvers, 2001; Bosch *et al.*, 2002; Cox *et al.*, 2003). In addition, analysis of the recently determined genome sequence of avian *P. multocida* identified numerous genes for putative hemoglobin and hemin binding proteins such as a heme acquisition system receptor (*hasR*) homologue, suggesting that *P. multocida* might also use free hemin as an iron source. Furthermore, as deduced from the genome sequence, a ferric uptake regulator (*fur*) is also present in *P. multocida*, indicating that a Fur repressor mode operating system could be responsible for iron regulation (May *et al.*, 2001)

It has been hypothesized that outer membrane proteins that are expressed in vivo under iron-restricted conditions might be involved in protective immunity against *P. multocida* (Adler *et al.*, 1996). Several studies performed in mice, chickens, turkeys, rabbits and cattle have shown that vaccination with *P. multocida* IROMPs stimulates protective immunity against experimental challenge (Glisson *et al.*, 1993; Kennett *et al.*, 1993; Ruffolo *et al.*, 1998; Srivastava, 1998; Confer *et al.*, 2001). In contrast, vaccination with a recombinant *P. multocida* IROMP (HgbB) did not protect mice against challenge, and insertional inactivation of the *hgbB* gene did not affect the ability of *P. multocida* to bind hemoglobin or its ability to produce disease in the mouse model (Cox *et al.*, 2003).

In the studies reported herein, we investigated the antibody response of cattle to IROMP-enriched fractions of *P. multocida* A:3 and correlated these responses with immunity to experimental challenge. We further identified an IROMP recognized by vaccinated cattle as the most immuno-dominant antigen. Protein sequence analysis identified this IROMP as a HasR homologue, which was subsequently confirmed by use of genomic tools.

Materials and methods

Bacterial strains, plasmids and culture conditions

Lyophilized cultures of *P. multocida* A:3 strain 232 (Pm232), originally isolated from a bovine pneumonic lung, were grown on brain-heart infusion agar (BHI DIFCO Labs, Sparks, MD) containing 5% bovine blood or BHI broth as previously described (Confer *et al.*, 1996). For production of IROMPs, the iron chelator 2, 2-dipyridyl (Sigma Chemical Co, St Louis, MO) was added to BHI broth at a final concentration of 0.150 mM prior to inoculation with *P. multocida* (Gilmour *et al.*, 1991). Other *P. multocida* strains (serogroups A, B and D and serotypes 1-18) were grown as described (Confer *et al.*, 1996).

Escherichia coli TOP10 and BL21(DE3)pLysS One Shot® chemically competent cells (Invitrogen®, Carlsbad, CA) were cultured in Luria-Bertani (LB) broth or on LB agar (DIFCO Labs, Sparks, MD). *E. coli* strains harboring plasmids were cultured with the addition of ampicillin at a concentration of 100 μg/ml and/or chloramphenicol at 35 μg/ml. Plasmid pCR®-XL-TOPO (Invitrogen®) was used as a standard cloning vector.

Bacterial outer membrane preparation

Bacterial outer membranes were prepared by sonication and centrifugation as previously described (Simons *et al.*, 1992). Outer membranes were extracted with 0.5% sodium N-laurylsarcosine (Sarkosyl; Sigma) in 0.01 Tris buffer and the insoluble fraction collected by centrifugation as previously described (Squire *et al.*, 1984; Dabo *et al.*, 1997). These outer membrane preparations are referred to as OMP-enriched or IROMP-enriched fractions. For vaccination, OMP- and IROMP-enriched fractions were resuspended in sterile phosphate-buffered saline (PBS) at a concentration of 2 mg/ml.

Animal studies

Experiment #1: To determine the antibody response of cattle to OMPs and IROMPs, 24 yearling crossbred beef heifers were randomly divided into 3 groups of eight heifers each (Control, OMPs and IROMPs). Heifers were vaccinated subcutaneously on days 0 and 14. Each vaccine contained 1 ml of Freund's incomplete adjuvant (FIA) and 1 ml of PBS, OMP- or IROMP-enriched fraction (at a concentration of 2 mg/ml). Serum samples were collected on days 0, 7, 14, 21, 27 and 35 and stored at -70°C for analysis at a later date.

Experiment #2: To determine if OMPs or IROMPs could enhance resistance to challenge, 24 crossbred beef cattle were randomly divided into four groups (Control, n=7; OMPs, n=7; IROMPs, n=7; and LIVE, n=3). Each group was vaccinated as described in the first experiment with the exception that the LIVE group received 2 x 109 colony forming units (CFU) of live *P. multocida* diluted in PBS. Serum samples were collected from all cattle on days 0, 3, 7, 10, 14, 17, 21 and 27. On day 27, cattle were challenged transthoracically with 5 x 10^9 CFU of *P. multocida* (Panciera and Corstvet, 1984). Four

days later, cattle were humanely euthanized and the lungs recovered. Lung lesion scores (20 being the maximum score) were determined as previously described (Panciera *et al.*, 1984).

Enzyme-linked immunosorbent assay (ELISA)

Antibody responses to OMPs and IROMPs of *P. multocida* were determined by an ELISA as previously described, with minor modifications (Confer *et al.*, 1996). Briefly, wells of 96-well microtiter plates (COSTAR #9018, Corning Inc., Corning, NY) were coated with 100 ng of either OMP- or IROMP-enriched *P. multocida* antigen. Primary antisera were diluted 1:800 in PBS containing 0.5% Tween 20 and 1% bovine serum albumin (BSA). The bound antibody was then detected using a 1:400 dilution of phosphatase-labeled affinity-purified goat anti-bovine IgG H+L (Kirkegaard & Perry Laboratories [KPL], Gaithersburg, MD). Enzymatic activity was assayed using ρ-nitrophenylphosphate. Antibody responses are expressed as nanograms of IgG bound based on a standard curve for each plate.

Electrophoresis and immunoblot analysis

Whole cell lysates and/or IROMP-enriched fractions were equilibrated to 1 mg/ml protein and subjected to discontinuous SDS-PAGE on 10% gels (Mosier *et al.*, 1989). Gels were stained with Coomassie brilliant blue R-250 for protein detection. For Western blots, proteins were transferred to a nitrocellulose membrane by the method of Towbin *et al.* (1979), at 100 V for 1 hour. Antigens were identified immunologically, using a 1:50 dilution of bovine sera. Immune complexes were detected with an affinity-purified biotin-labeled goat anti-bovine IgG H+L followed by phosphatase-labeled streptavidin (KPL) (Mosier *et al.*, 1989). The alkaline phosphatase substrate used was 5-

bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium. Immunoblots were further analyzed by densitometry using a video densitometer in reflectance mode (Mosier *et al.*, 1989). Analyses of peaks were performed and data were expressed as total peak area optical density for each band.

***P. multocida* gene cloning and DNA analyses**

Chromosomal DNA was isolated from *P. multocida* as previously described (Ausubel *et al.*, 1987). The *hasR* gene was amplified from genomic DNA by PCR as follows: The PCR mixture consisted of 20 ng of genomic DNA, 15 pmol of primers designed from published *P. multocida* sequence data, 1.5 mM MgCl2, and 2.5 U of Taq DNA polymerase (Platinum® Pfx, Invitrogen, Carlsbad, CA) in 50 µl of reaction buffer (Table 2) (May *et al.*, 2001). PCR amplifications were performed in a Gene Amp PCR system 2400 (Perkin-Elmer Cetus Inc., Norwalk, CT) using the following conditions: initial denaturation at 94°C for 2 min, then 30 cycles (denaturation 94°C for 30 sec, annealing at 55°C for 1 min and elongation at 72°C for 3 min), followed by a final elongation at 72°C for 7 minutes. PCR fragments were gel-purified and cloned into the pCR®-XL-TOPO vector (Invitrogen® Carlsbad, CA). Plasmid DNA was isolated using Wizard® Plus Minipreps DNA Purification System (Promega Corp., Madison, WI). Cloned DNA inserts in recombinant plasmids were sequenced at the OSU Recombinant DNA/Protein Resource Facility on an Applied Biosystems 373A automated DNA sequencer (ABI, Foster City, CA). DNA sequences were assembled and analyzed with MacVector/ Assemblylign 6.5 sequence analysis software (Accelrys, San Diego, CA). Protein profiles were performed using MacVector. The deduced amino acid sequence of

hasR was compared with other sequences in GenBank using BLAST or FASTA, and alignments were generated with MacVector (Altschul *et al.*, 1990).

Hemin-binding blot

P. multocida A:3 whole cell lysates and OMPs expressing or devoid of IROMPs were solubilized in Laemmli sample buffer (LSB) and separated by SDS-PAGE (10% [wt/vol] acrylamide) and the gel stained with Coomassie brilliant blue. For hemin-binding blots, gels were transferred to nitrocellulose membranes as previously described (Towbin *et al.*, 1979; Carroll *et al.*, 2000). The blotted membrane was rinsed with Tris-buffered saline (TBS; 10 mM Tris-HCl [pH 8.0] containing 150 mM NaCl) containing Tween 20 (0.1% [vol/vol]) and probed with TBS containing hemin (10μg/ml) for 1.5 h. The hemin-binding blot was then rinsed three times for 30 min with TBS-Tween 20 and developed using enhanced chemiluminescense (ECL) reagents (Amersham Pharmacia, Piscataway, NJ). Hemin-binding protein bands were visualized autoradiographically.

HasR surface exposure determination

Surface exposure of HasR was determined by immunoblotting using surface antibodies. Bovine immune serum was absorbed with intact *P. multocida* as previously described with some minor modifications (Pandher *et al.*, 1998). Briefly, 1 Lt. cultures of *P. multocida* expressing IROMPs in logarithmic phase (A_{600} of 0.5) were pelleted by centrifugation, washed twice in PBS, and resuspended in serum from a rabbit vaccinated with gel-purified HasR from *P. multocida* Pm232 diluted 1:100 in Tris-saline-nonfat dry milk (TSM) (10 mM Tris [pH 7.4], 0.9% [wt/vol] NaCl, 1% nonfat dry milk). Cells resuspended in serum were incubated at 4°C for 4 hours on a rocking platform. This process was repeated three times using a new bacterial pellet each time. Following

incubation, the cells were pelleted by centrifugation at 11,000 X g. The supernatant was carefully removed and stored at -20°C. Surface antibodies were eluted from the cell pellets in [3.0] glycine. Western immunoblots were performed using whole-cell lysates of *P. multocida* to determine the presence of surface exposed antigens. The unabsorbed serum was used as a positive control and was diluted 1:100 in TSM before use. The adsorbed serum (supernatant) was used undiluted as the negative control. The eluted fraction was used undiluted as well.

Preparation of rabbit immune sera against *P. multocida* HasR

Two *Pasteurella*-free New Zealand white male rabbits weighing 2.5 kg were purchased from Harlas Sprague Dawley (Indianapolis, IN). The nasal cavities of both rabbits were determined to be free of *P. multocida* by performing serial bacterial cultures on day 0 and each subsequent time they were handled for the duration of the experiment. Both rabbits were also determined to be negative at day 0 for serum IgG against *P. multocida* whole cells by western immunoblotting. Each rabbit received approximately 250 μg of gel-purified HasR subcutaneously on days 0 and 14. Briefly, a known concentration of *P. multocida* IROMP-enriched fractions was separated by SDS-PAGE using a 10% gel. The isolated HasR band was identified, cut out of the gel and purified using established methods (Gatto *et al.*, 2002). Sera were collected weekly for 6 weeks and stored at -20°C until used. Development of an immune response to *P. multocida* HasR was monitored by western blotting using rabbit sera at a 1:100 dilution. Immune complexes were detected with an affinity purified phosphatase-labeled goat anti-rabbit secondary antibody as described above.

Statistical Analysis

Comparisons of mean antibody responses to *P. multocida* OMPs and IROMPs were made between treatment groups by PROC MIXED analysis with repeated measures (SAS System 8.02 TS Level 02M0). Comparisons were also made of serum antibody responses to individual *P. multocida* IROMPs (96 and 107 kDa bands) within groups (Experiment #1 days 0 and 35 and experiment #2 days 0 and 27). Linear regression analyses were done to correlate lung lesion scores with antibody responses to *P. multocida* OMPs or IROMPs as measured by ELISA or individual IROMPs as measured by densitometry (Confer *et al.*, 1995).

Results

Iron-induced outer membrane protein expression

Outer membrane fractions from *P. multocida* grown in media under iron-sufficient or iron-deficient conditions were subjected to SDS-PAGE to compare their protein profiles. The OMP profiles from cultures containing the iron-chelator 2,2-dipyridyl expressed four IROMPs with approximate molecular weights of 48, 80, 96 and 107 kDa. These proteins were not present in outer membrane fractions of bacteria grown under iron-sufficient conditions (Fig. 5). In order to determine if these IROMPs were expressed in vivo, western immunoblotting was performed and probed with serum from a cow immunized against *P. multocida* (Fig. 5). Four major IROMPs (48, 78, 80 and 96 kDa) were recognized.

Animal experiments

Experiment 1 – We determined the immunogenic potential of OMPs and IROMPs in cattle. Antibody responses of cattle to *P. multocida* OMPs or IROMPs were measured by

ELISA. Vaccination of cattle with OMP or IROMP-enriched fractions resulted in a significant ($P<0.01$) increase in serum antibody responses to *P. multocida* OMPs and IROMPs by 14 days after vaccination (Fig. 6), with the highest mean antibody concentration being recorded on day 21 post vaccination. Heifers vaccinated with IROMP-enriched fractions had significantly ($P<0.01$) higher mean antibody concentrations to *P. multocida* OMPs and IROMPs on days 14 and 21 than did heifers vaccinated with the OMP-enriched fraction. Results obtained from OMP and IROMP ELISA parallel each other.

Of the four major IROMPs (48, 80, 96 and 107 kDa), only two prominent antigenic bands (96 and 107 kDa) were recognized by western blotting and could be quantified by densitometry. Vaccination with OMP- or IROMP- enriched fractions caused a significant ($P<0.05$) increase in antibody binding to the 96 kDa protein band between days 0 and 35 (Fig.7). Antibody responses to the 107 kDa band were inconsistent and no significant difference was found in antibody binding to this band.

Experiment 2 – A second experiment was conducted to determine the protective capacity of anti-OMPs or anti-IROMPs against experimental *P. multocida* challenge. Serum antibody responses to *P. multocida* OMPs and IROMPs as measured by ELISA were higher in this experiment than in experiment 1.

Mean serum antibody concentrations against OMPs increased significantly ($P<0.03$) 14 days after vaccination with live *P. multocida* and remained significantly elevated for the duration of the study (Fig. 8). In addition, cattle immunized with OMPs or IROMP-enriched fractions had a significant ($P<0.005$) increase in antibody responses to *P. multocida* OMPs on day 27 after vaccination. Mean serum antibody concentrations were

significantly ($P<0.05$) higher on day 21 after vaccination of cattle with live *P. multocida* than for cattle vaccinated with IROMP-enriched fractions.

Mean serum antibody concentrations to IROMPs increased significantly ($P<0.001$) 21 days after vaccination with live *P. multocida* and remained significantly elevated for the duration of the study (Fig. 8). Vaccination with live *P. multocida* and IROMP-enriched fractions resulted in significantly ($P<0.04$) higher mean antibody concentrations 21 days after vaccination than in cattle immunized with OMP-enriched fractions. Cattle vaccinated with IROMP-enriched fractions had a significant ($P<0.03$) increase in mean antibody concentrations at 14 days, which remained significantly increased through the duration of the study. Vaccination of cattle with IROMP-enriched fractions induced significant ($P<0.01$) increases in mean antibody responses on day 21 that were greater than antibody responses for cattle immunized with OMP-enriched fractions. At day 27 after vaccination, cattle immunized with OMP-enriched fractions had a significant ($P<0.001$) increase of mean serum antibody concentrations.

There was a significant ($P<0.05$) increase in mean antibody binding to the 96 kDa IROMP band between days 0 and 27 in cattle vaccinated with either the IROMP-enriched fraction or live *P. multocida* (Fig. 9). There were no significant differences ($P>0.05$) in antibody responses between days 0 and 27 for the control and OMP groups or between the treatment groups. In addition, antibody responses to the 107 kDa band were not significantly different between days 0 and 27 (data not shown).

Vaccination with PBS was associated with significantly higher ($P<0.05$) mean lung lesion scores (12.86 ± 3.37) than for cattle vaccinated with live *P. multocida* (1.83 ± 0.33), OMP (4.42 ± 0.68) or IROMP-enriched fractions (4.92 ± 1.29). There were no

significant differences (P>0.05) in mean lung lesion scores among the Live, OMP- or IROMP-enriched fraction groups.

The correlation between lung lesion score and mean serum antibody concentrations as measured by ELISA for *P. multocida* OMPs and IROMPs was not significant (r = -0.36 P = 0.07 and r = -0.34 P = 0.1 respectively). However, the correlation between lung lesion scores and antibody responses to the 96 kDa protein band approached significance (r = -0.4 P = 0.0558).

Cloning and characterization of *hasR*

Since the 96 kDa IROMP appeared to be a major target the 96 kDa IROMP band was cut from a gel and subjected to mass spectrometry analysis (Yale University). The resulting sequences suggested that a homologue of the iron-regulated protein HasR might be present in the sample (Table 3).

The *hasR* gene was amplified from genomic DNA of *P. multocida* Pm232 using oligonucleotide primers (Table 2) designed from the *P. multocida* Pm70 genome sequence (May *et al.*, 2001). The 2.5-kb fragment obtained was cloned into the pCR®–XL-TOPO vector and sequenced to confirm that no mutation was introduced during the amplification reaction. The cloned insert revealed an open reading frame of 2,547 nucleotides that begins with an ATG codon and encodes a protein with a calculated molecular mass of 95.9 kDa and a pI of approximately 9.42 (Fig. 10). A predicted secretory signal cleavage site was found in the N-terminus between residues S26 and Q27. Upstream from the start codon there is a potential ribosome-binding site (GGAGAA) with 85% identity to the ribosomal binding site consensus sequence (Steitz and Jakes, 1975). In addition, a well-conserved Fur box (GTTAATGATTTTCATTATC)

was found 31-bp upstream of the start codon, with 84.2% identity to the Fur consensus sequence (de Lorenzo *et al.*, 1987; Escolar *et al.*, 1999). The predicted protein has a TonB box in its N-terminus located at amino acid position 38 (ETILVNES) similar to the ones found in TonB-dependent iron receptors (Tuckman and Osburne, 1992). A search of GenBank sequences and subsequent sequence alignments revealed that the deduced amino acid sequence of the Pm232 HasR homologue has 98% identity with the published sequence of HasR from Pm70. Differences between the sequences of Pm70 and Pm232 consisted of 28 nucleotides (4%). These changes resulted in only 10 amino acid differences, 8 of which were conservative substitutions (Table 4 and fig. 10). In addition, the *P. multocida* HasR has 25% and 23% identity with the HasR from *Pseudomonas aeruginosa* and *Serratia marcescens*, respectively. Transmembrane computer predictions suggested that the HasR protein is highly antigenic and has a high probability of numerous surface-exposed regions based on the hydrophilicity character of the protein (Fig. 11), a characteristic common to other HasR proteins.

Hemin-binding blot – A hemin-binding blot was performed to determine if hemin-binding proteins were present in OMP profiles of *P. multocida*. Eight major protein bands of molecular weights ranging from 35 to 107 kDa were found to bind hemin including 2 major IROMPs, one of them being HasR (fig. 12). Two of the protein bands (35 and 45 kDa) showed heat modifiability. However, HasR was not heat modifiable.

Surface exposure – To determine if HasR is surfaced exposed on the outer membrane of the bacterium, we performed western immunoblots probed with serum from a rabbit that was immunized with *P. multocida* HasR. Absorption of rabbit immune serum with intact *P. multocida* expressing IROMPs did not result in a loss of antibody reactivity to

HasR on western immunoblots (Fig 13). In contrast, eluted antibodies from the intact bacterium showed loss of reactivity to HasR. The results of this experiment suggest that HasR is not surface exposed (Fig. 13).

Conservation of HasR among serotypes and/or serogroups of P. multocida – To determine if HasR is conserved among other *P. multocida* serotypes and/or serogroups, we attempted to amplify the *hasR* gene by PCR from genomic DNA of 21 isolates obtained from different animal species. The hasR gene was amplified and identified in 12 out of the 21 isolates (Fig. 14). No amplification was observed when chromosomal DNA from *Mannheimia haemolytica* or *Haemophillus somnus* was used.

Discussion

In recent years, research has focused in identifying essential virulence factors for pathogenic bacteria that are expressed in vivo as potential targets for vaccine development. Expression of IROMPs by many pathogens, including *P. multocida*, could play an important role in virulence, making these proteins an attractive target for vaccine design. Vaccination with IROMPs has been shown to enhance resistance in several *P. multocida* animal models (Glisson *et al.*, 1993; Kennett *et al.*, 1993; Ruffolo *et al.*, 1998; Srivastava, 1998; Confer *et al.*, 2001). In addition, administration of purified *M. haemolytica* IROMPs afforded protection against challenge in vaccinated cattle (Potter *et al.*, 1999). Results of the present study show that vaccination of cattle with live *P. multocida* or IROMP-enriched fractions stimulated significant antibody responses to a major 96 kDa IROMP. Based on sequence analysis, we identified this protein as a HasR homologue. Furthermore, vaccination of cattle with OMP- or IROMP-enriched fractions resulted in enhanced resistance to experimental challenge. However, vaccination of

cattle with IROMP-enriched fractions did not offer any advantages over vaccination with OMPs.

In this study, we used the transthoracic inoculation method as the challenge route (Panciera and Corstvet, 1984). This method, although effective in inducing consistent and reproducible lung lesions, is unnatural as the direct placement of the bacteria in the lungs bypasses upper respiratory tract defense mechanisms as well as the secretory immunity of the pulmonary bronchioles. Local immune responses might be important in preventing infection with *P. multocida*. Confer *et al.* (2001), previously showed that intranasal vaccination of rabbits with *P. multocida* IROMP-enriched fractions enhanced nasal bacterial clearance when compared to controls. Therefore, it might be possible to enhance resistance of cattle to challenge with *P. multocida* by delivering IROMP-enriched fractions intranasally so as to stimulate local immune responses such as secretory IgA. Furthermore, Potter *et al.* (1999), evaluated the efficacy of purified IROMP (TbpA+TbpB) vaccination in cattle by utilizing an aerosol challenge method. They administered bovine herpesvirus-1 by aerosol followed by *M. haemolytica* 4 days later. This challenge method more closely resembles the conditions of natural infection. These investigations showed that vaccination with recombinant *M. haemolytica* IROMPs (TbpA+TbpB) correlated with lower clinical scores and, therefore, enhanced protection of cattle against challenge when compared to TbpA-, TbpB- or PBS-vaccinated groups. Thus, vaccination with IROMPs using different challenge methods has demonstrated the induction of protective immunity.

Vaccination with IROMP-enriched fractions did not provide any added resistance to challenge over vaccination with OMP-enriched fractions. A potential explanation for

these results might be that the OMP-enriched fractions are contained in the IROMP preparation, and the iron-induced OMPs account for only about 3% of the total IROMP fraction. Therefore, in order to see significant differential effects, a dose titration study would be necessary where OMP-enriched fractions containing different concentrations of purified IROMPs would be tested.

To enhance the immune response to the OMP-enriched fractions, we used Freund's incomplete adjuvant (FIA). This adjuvant is relatively inexpensive and widely used in research. The mechanism of action, as for other water-in-oil type adjuvants, is stimulatory of humoral but not a cellular immune response, acting as a depot at the site of injection or in antigen presenting cells (Warren *et al.*, 1986). Similarly, other studies examining the immunogenic potential of *P. multocida* IROMPs have used FIA or other emulsion adjuvants such as parafilm-lanolin to enhance the immune response (Glisson *et al.*, 1993; Ruffolo *et al.*, 1998; Srivastava, 1998; Borkowska-Opacka and Kedrak, 2002). In contrast, Confer *et al.* (2001), used the B-subunit of cholera toxin (CT) to stimulate a cellular as well as humoral immune response to *P. multocida* IROMPs in rabbits. In that study, rabbits vaccinated with IROMPs-CT had significantly higher serum and nasal antibody responses to *P. multocida* and significantly lower nasal bacterial counts following intranasal challenge than control rabbits. Therefore, a different type of adjuvant that stimulates cellular as well as the humoral immune response might be preferable over FIA for assessing the potential use of *P. multocida* IROMP-enriched fractions as immunogens to stimulate protection against infection of the respiratory tract.

We identified a highly immunoreactive band of 96 kDa on immunoblots of *P. multocida* IROMPs that were probed with sera from vaccinated cattle. A correlation

between anti-96 kDa antibodies and low lesion scores was made that approached significance (P=0.055). It was therefore decided to further characterize this protein. As mentioned earlier, this protein was identified as a HasR homologue. HasR from *P. multocida* Pm232, ($HasR_{Pm232}$) shares a lot of similarities with other outer membrane proteins. First, a well-conserved Fur box was found upstream from the start codon, which supports iron-regulation in a Fur-dependent system. In addition, a TonB box was localized in the very N-terminus region, which shares homology with other TonB-dependent receptors. This protein is most likely a heme-binding protein as demonstrated by hemin-binding blot results. Sequence alignment showed that $HasR_{Pm232}$ and HasR from *S. marcescens* ($HasR_{Sm}$) share little identity, which might suggest that $HasR_{Pm232}$ is a distant relative of this type of hemin- or hemoglobin-binding receptor.

We tested a number of *P. multocida* serogroups and/or serotypes for the *hasR* gene by PCR. The *hasR* gene was readily amplified from 12/21 of strains tested. Although we did not amplify the *hasR* gene from 9/21 *P. multocida* strains, we consider it unlikely that these isolates do not have HasR. Although, the primers were located in highly conserved regions of the gene, it might be that the strains that did not amplify by PCR have slight genetic variations in the primer regions resulting in failure to amplify the *hasR* gene. Modifications of the PCR assay might improve the chances of amplifying this gene, such as the use of multiple oligos targeting different regions within the *hasR* gene or the use of degenerate primers. Alternatively, other less specific techniques such as Southern blotting could clarify whether these strains are truly lacking a *hasR* gene homologue. Even so, with the information gained from PCR screening we can estimate that a majority

of *P. multocida* strains probably express HasR since over 60% of the strains tested were PCR positive.

Results from computer analysis of the predicted amino acid sequence suggest that HasR is an outer membrane protein. As predicted by the hydropathy profile, HasR has several domains that probably extend into extracellular region, giving a high probability of it being surface exposed. HasR has a hydrophobic region at the amino-terminus that is characteristic of a signal peptide which would allow this protein to be transported across the inner membrane to its final destination, the outer membrane. Although these characteristics suggest that the HasR protein is surface-exposed, we could not demonstrate surface exposure of HasR by antibody adsorption. Several factors could have affected the outcome of this experiment. For example, there might have been enough residual capsular material to mask external HasR regions from antibodies. Also, the exposed domains of HasR may be very short and shielded by structures such as LPS or other OMPs, again preventing the interaction of antibodies with the HasR protein. In addition, surface exposed regions of HasR may not be highly immunogenic, and the antibodies against HasR that we found could have been primarily against other regions of the protein that were exposed to the immune system during membrane degradation. Therefore, antibodies against HasR might not play a role in protection where binding with live *P. multocida* is necessary for opsonization and/or complement-mediated killing.

To our knowledge, no other studies have directly assessed the immunogenic potential of HasR from any bacterial species. Although the antibody binding assay did not suggest surface exposure of this protein, it was shown to be immunogenic and a high antibody response to HasR correlated with lower lesion scores at slightly greater than

$P<0.055$. Therefore, further investigations are needed to clarify whether HasR is surface exposed and the role that antibodies against this protein might play in preventing infection with *P. multocida*.

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Table 2. Primers used in this study to amplify and sequence the *hasR* gene from *P. multocida* A:3 Pm232.

Primers	Sequence	Position
HasR F	5'-AATGTTATTTATTCACCTCTTGCATTTCTC-3'	
HasR R	5'-AATTCAAACATGCCTAATAATAGCCGAC-3'	
1F	5'-GGTCAATGAGAGTGAAGAG-3'	122
2F	5'-GAATGCATACAATTATAGCC-3'	731
3F	5'-GTCGATAGCTATCATGC-3'	1155
4F	5'-TATGATTATATTGTGAGA-3'	1596
5F	5'-GGTGACGCAGACAATTAATATAC-3'	2028
1R	5'-GGTACTAATCGCTTACTG-3'	2312
2R	5'-GTCGCACTCATGGACCAG-3'	1838
3R	5'-AGCTCTCACACGTGCT-3'	1436
4R	5'-GAACGCGAGGCTCTTTTG-3'	889
5R	5'-GTCTCTACTCTCACTACCCT-3'	498

Fig. 5. SDS-PAGE and western immunoblot comparing *P. multocida* A:3 outer membrane fractions expressing or devoid of IROMPs. (A) Coomasie blue-stained 10% SDS gel of outer membrane proteins of *P. multocida* A:3 grown under iron-sufficient (OMP) or iron-deficient conditions (IROMP). Lane 1 – OMP; Lane 2 – IROMP. (B) Western immunoblot of outer membrane proteins of *P. multocida* A:3. Lane 1 – IROMP; Lane 2 – OMP. Blots were treated with serum from a bovine (#589) that was immunized with IROMPs. The position of molecular weight standards in kDa run on the same gel are indicated. Major IROMPs are indicated by arrows.

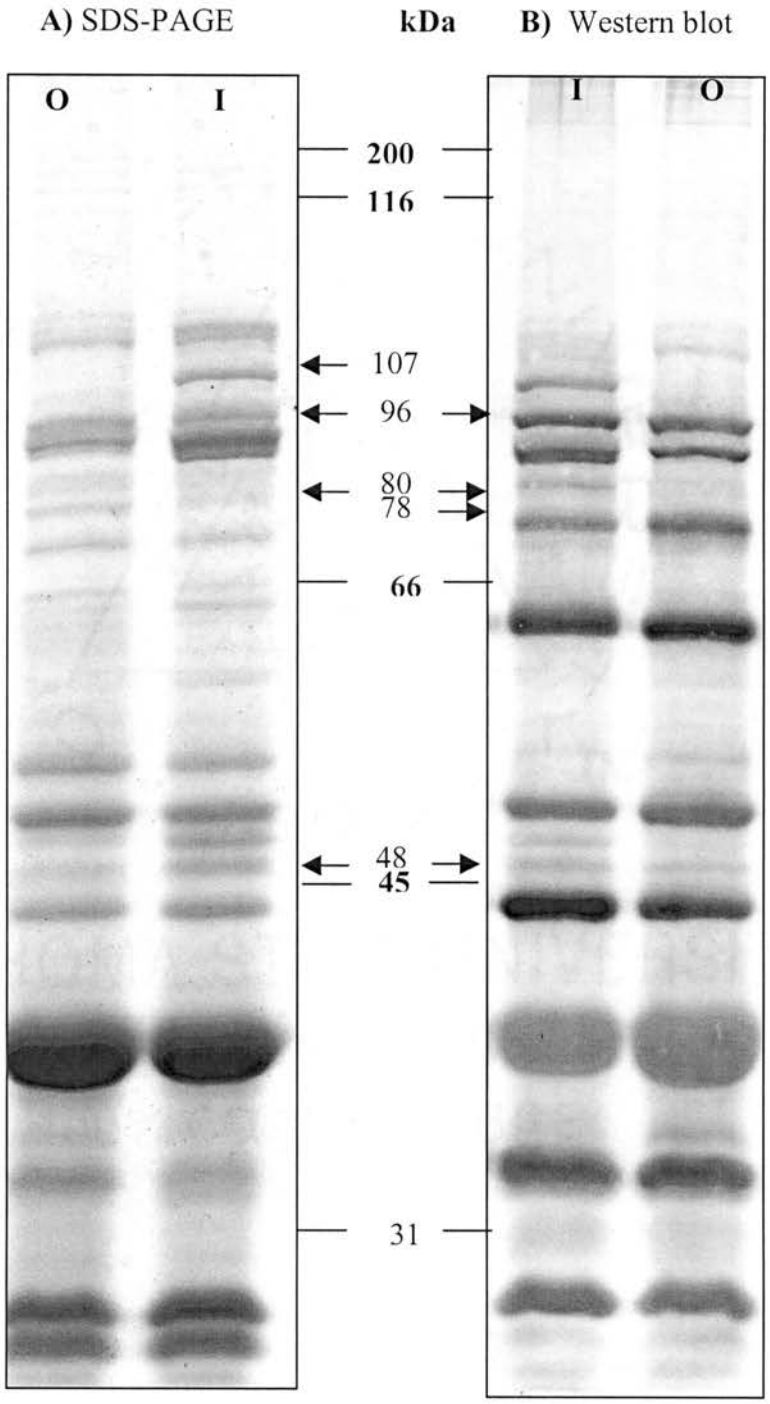
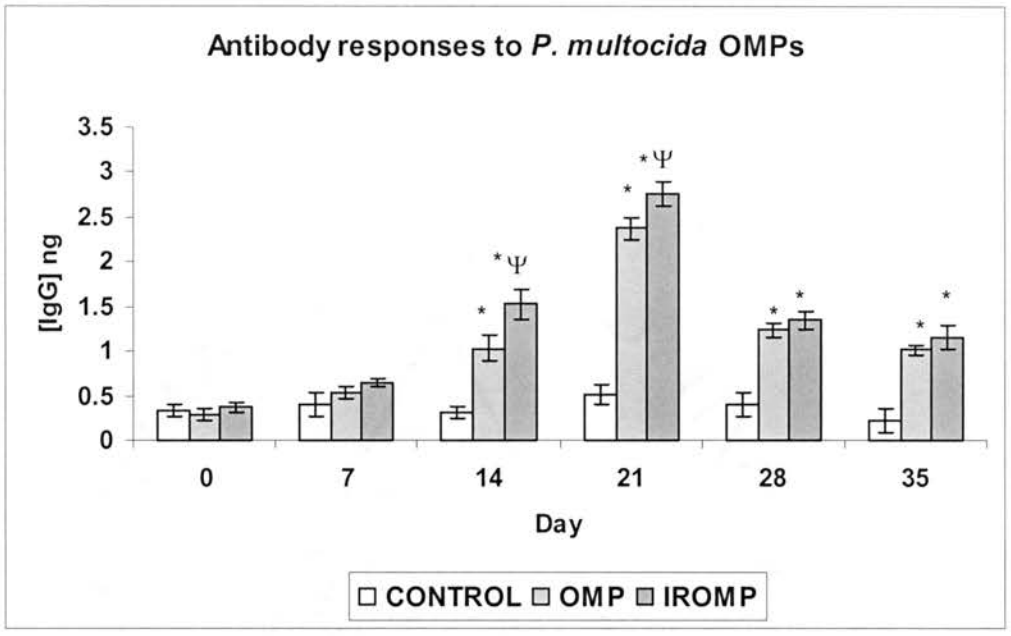

A) SDS-PAGE
kDa
B) Western blot
O
I
I
O
200
116
107
96
80
78
66
48
45
31

Fig. 6. Serum antibody responses of beef heifers on range pasture to OMPs and IROMPs of *P. multocida* A:3 following vaccination with PBS, OMPs or IROMPs as measured by ELISA. Data are expressed as nanograms/ of serum IgG. Data shown are the mean and standard error of the mean (SEM) for each group. A $P<0.05$ is considered significant. Differences (*) from control or (Ψ) between treatment groups are noted.


Antibody responses to P. multocida OMPs
[IgG] ng
3.5
3
2.5
2
1.5
1
0.5
0
0
7
14
21
28
35
Day
CONTROL
OMP
IROMP

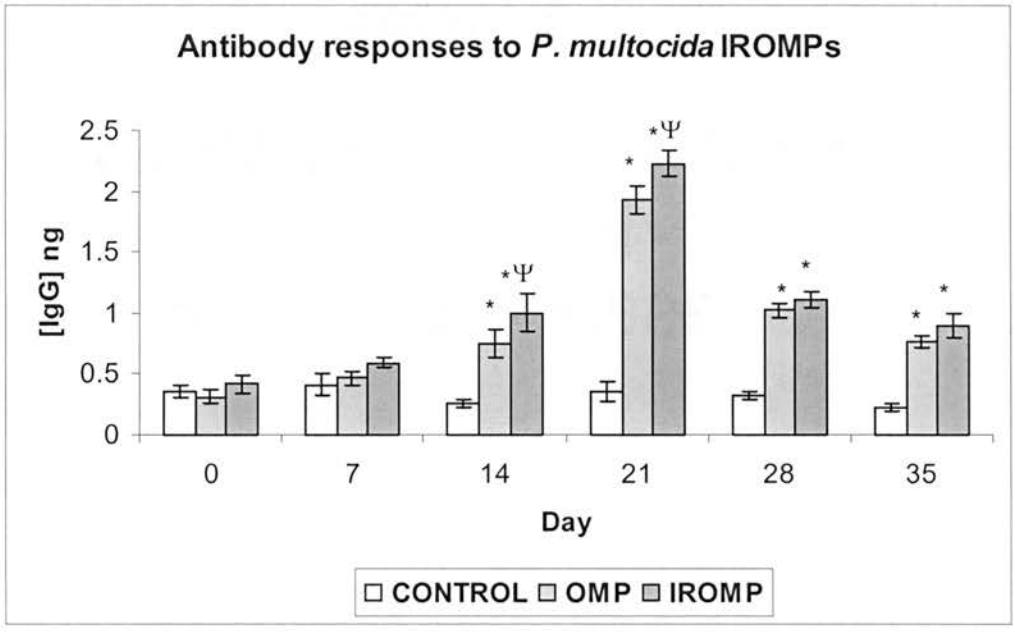
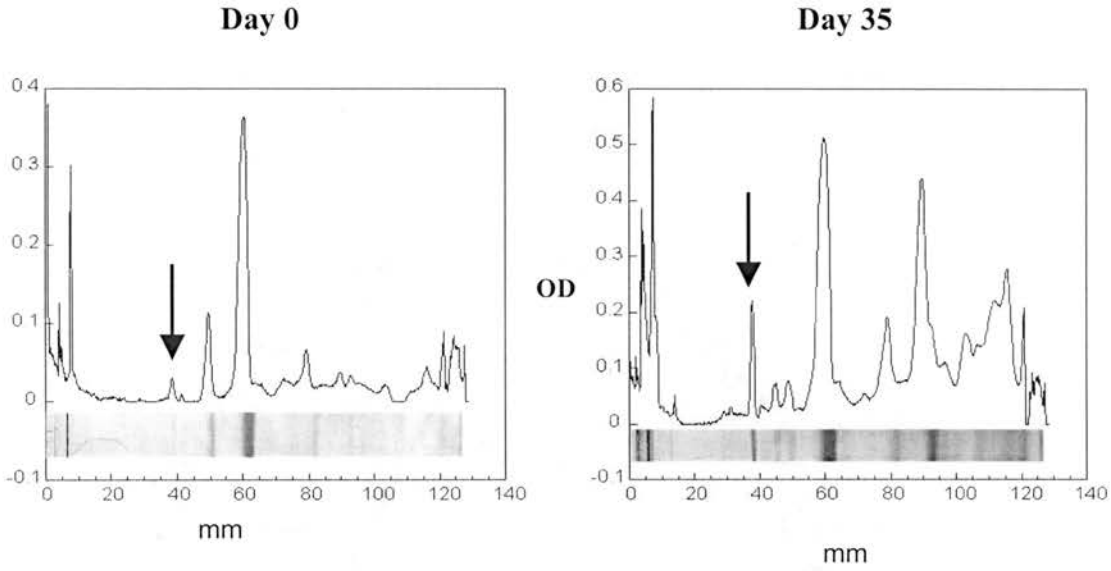

Antibody responses to P. multocida IROMPs
[IgG] ng
2.5
2
1.5
1
0.5
0
0
7
14
21
28
35
Day
CONTROL
OMP
IROMP

Fig. 7. Densitometry results of western immunoblots demonstrating antibody responses of beef heifers to the 96 kDa protein on days 0 and 35. **Top:** Western immunoblot and densitometer scan profile demonstrating antibody responses from a cow (#287) to *P. multocida* A:3 pre (day 0) and post (day 35) immunization with IROMPs. The arrow depicts the peak corresponding to the 96 kDa protein (HasR). **Bottom:** Mean ± SEM Densitometry OD readings of western immunoblots from beef heifers vaccinated with PBS, *P. multocida* OMPs or IROMPs. A $P < 0.05$ was considered significant. Equal letters indicate NSD.

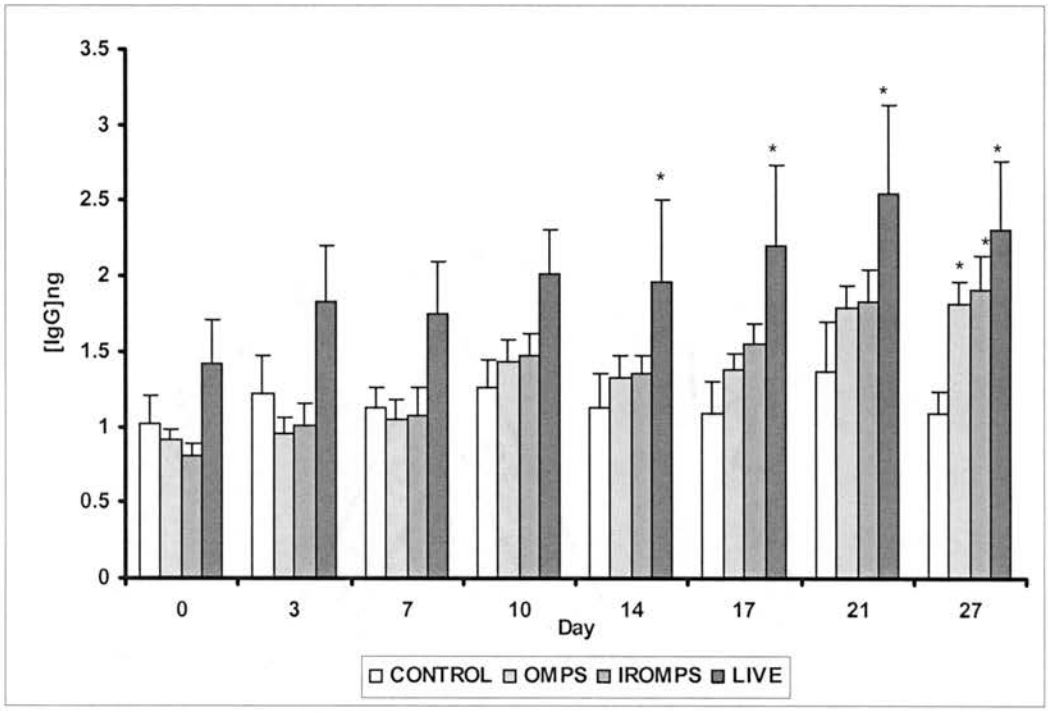


Mean ± SEM Densitometry OD readings

Group	Day 0	Day 35
Control	0.107 ± 0.028 ab	0.082 ± 0.027 a
OMPs	0.168 ± 0.420 b	0.240 ± 0.056 c
IROMPs	0.196 ± 0.069 b	0.309 ± 0.109 c

Fig. 8. Serum antibody responses of beef cattle to OMPs and IROMPs of *P. multocida* A:3 following vaccination with PBS, OMPs, IROMPs or Live bacteria as measured by ELISA. Data are expressed as nanograms of serum IgG. Data shown are the mean and standard error of the mean (SEM) for each group. A $P<0.05$ is considered significant. Differences (*) from control or (Ψ) between treatment groups are noted.

Antibody responses to *P. multocida* OMPs



Antibody responses to *P. multocida* IROMPs

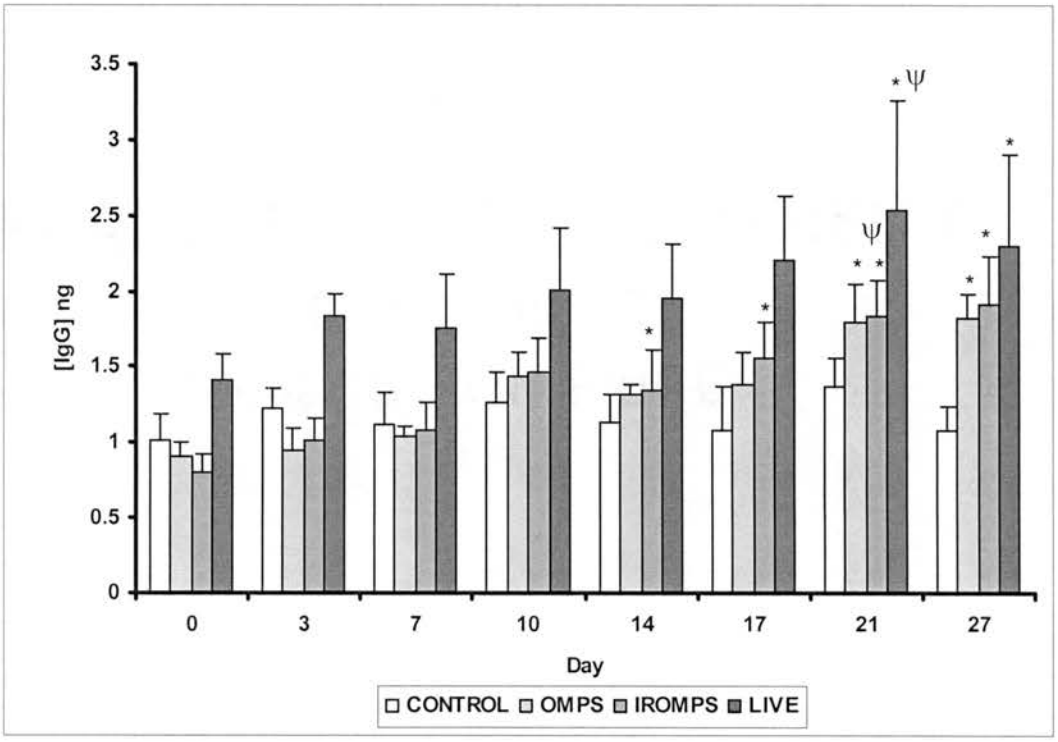
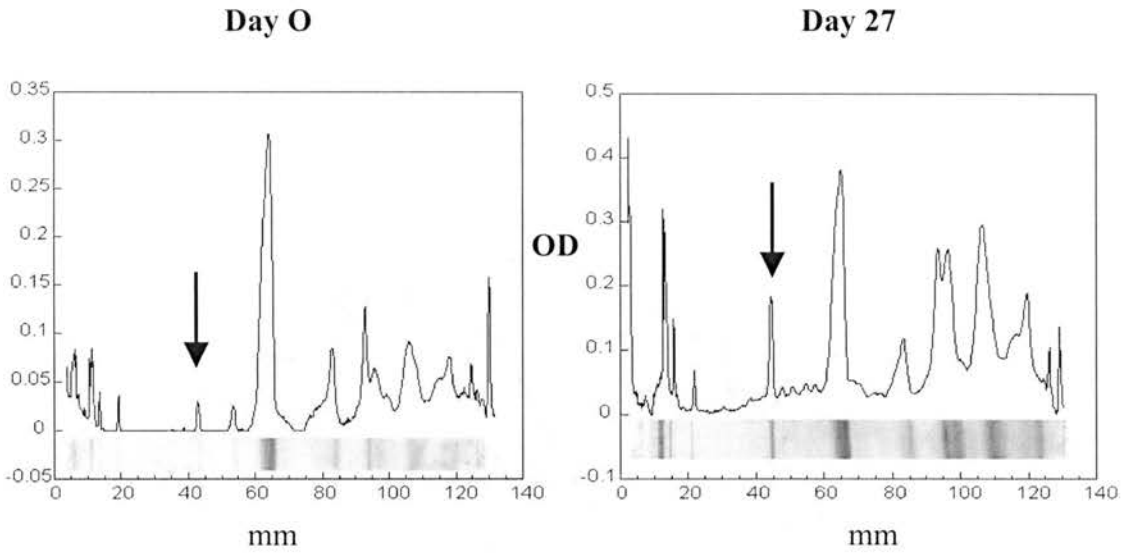


Fig. 9. Densitometry results of western immunoblots demonstrating antibody responses of beef heifers to the 96 kDa protein on days 0 and 27 (pre-challenge). **Top:** Western immunoblot and densitometer scan profile demonstrating antibody responses of a cow (# 3007) to *P. multocida* A:3 pre (day 0) and post (day 27)-immunization with IROMPs. The arrow depicts the peak corresponding to HasR. **Bottom:** Mean ± SEM Densitometry OD readings of western immunoblots from beef cattle vaccinated with PBS, Live *P. multocida,* OMPs or IROMPs. A $P < 0.05$ was considered significant. Equal letters indicate NSD.



Mean ± SEM Densitometry OD readings

Group	Day 0	Day 27
Control	0.209 ± 0.029 a	0.166 ± 0.033 ab
OMPs	0.088 ± 0.023 ad	0.237 ± 0.026 bcd
IROMPs	0.114 ± 0.043	0.280 ± 0.110 c
LIVE	0.161 ± 0.055 a	0.255 ± 0.052 ac

Table 3. Summary of *P. multocida* Pm232 HasR amino acid sequence fragments generated from MSMS Spectra. Pm232 HasR amino acid sequence (bottom) depicting the fragments generated from MSMS Spectra shaded in gray.

Amino acid position	Sequence
121	GLQSVPSYQGYAGSSTR
191	LGTMTNTVSPPPYYTR
256	WQNADLVLAYAK
485	GYPEDAIGPLYIR
526	YLQSTIYDYIVR
674	DKVTQTINIQSAQFK
811	LTMTMDNVFNR
822	YYLDINNMGLNTAPGR

MQKQQPYPIH LGIFLMLGLP TWAFSQANLE KSTINKLETI LVNESEEKNK
FDENLIKTYL SSGSYSYLSQ SDISTFRGSS VGDFLSGVPG VIVGNKRNSG
ALSVNIRGIA NENRVPVWID KGLQSVPSYQ GYAGSSTRTY LDPDLISQVE
IEKGPSLQMD ATGATGGVVR VETLRWQDII PQGKNWGVRL KLGTMANTVS
PPPYYTRGGY QTKYISKCLS NHTGLCQTQT YAPNARYSSH GFDLNAYNYS
LAFANKWQNA DLVLAYAKRK QGNYFVGRHG QTPVIESIEF EEDSVEVKEP
RVHEDVEIGS LTFKENRSTL YRPGEEALNT SQDNTSYLAK INVYNDVHRL
GLAYRHYHSR FGEIMSSILN FRAYGALQGE GTEVKVDSYH ANYSYNSTTP
YVNLSVNAYF TDSDSSNFTP FIEEYGYSLS SRHAHFLVSK QKGLSIENTS
IFQLNDKPFT LKYGLAHSYE RIYQPRNAQA RVRAKGYPED AIGPLYIRDG
KRKEWSAFVA ANYPITSWLK ADIGLRYLQS TIYDYIVRTE RVNIGGALVP
NPNGSGNIWV EKYKDVVHKQ APVKNKGMSP IVMFTFEPIN GVQIYTKYAE
ALRSPSLFQA TKGWSMSATA DNLEQLRPER AKNWEAGINL FYENLGGKDN
ILGFKLAYFN NRIKDYLTRS YSPKDKVTQT INIQSAQFKG IELSAYYDMG
KFYAKLAGTY YTKTKFCLTA EQAGKGEQCN SGYVYRSNLN NAVPPRLNLH
ATLGTRLFEQ KLDIGARYSY YSKRLVPVLS AERFVNTSSI EWAPYSLVDL
YANYNVSNNL KLTMTMDNVF NRYYLDINNT GLNTAPGRTL HLGLEYRF

Table 4. Nucleotide differences between sequences of Pm70 and Pm232 that resulted in amino acid changes. The amino acid encoded is listed and the nucleotide changes are noted with bold font.

	Pm70		Pm232	
Amino acid Position	Nucleotide	Amino acid	Nucleotide	Amino acid
197	ACT	T	**G**CT	A
305	GAG	E	GA**T**	D
397	CCA	P	**T**CA	S
405	AAT	N	A**G**T	S
460	AGT	S	A**C**T	T
549	TTT	F	**C**TT	L
556	CCC	P	**T**CC	S
585	CTC	L	**T**TC	F
734	ATA	I	**G**TA	V
830	ATG	M	A**C**G	T

Fig. 10. Nucleotide sequence of *P. multocida* A:3 *hasR* and the deduced amino acid sequence of HasR. The location of the putative Fur box and ribosomal binding site (RBS) are underlined. The start and stop codons, and signal peptide cleavage site are noted in bold font. The putative TonB box is depicted in a box. Differences between published genome sequence Pm70 and *P. multocida* A:3 nucleotides and amino acids are noted in bold font.

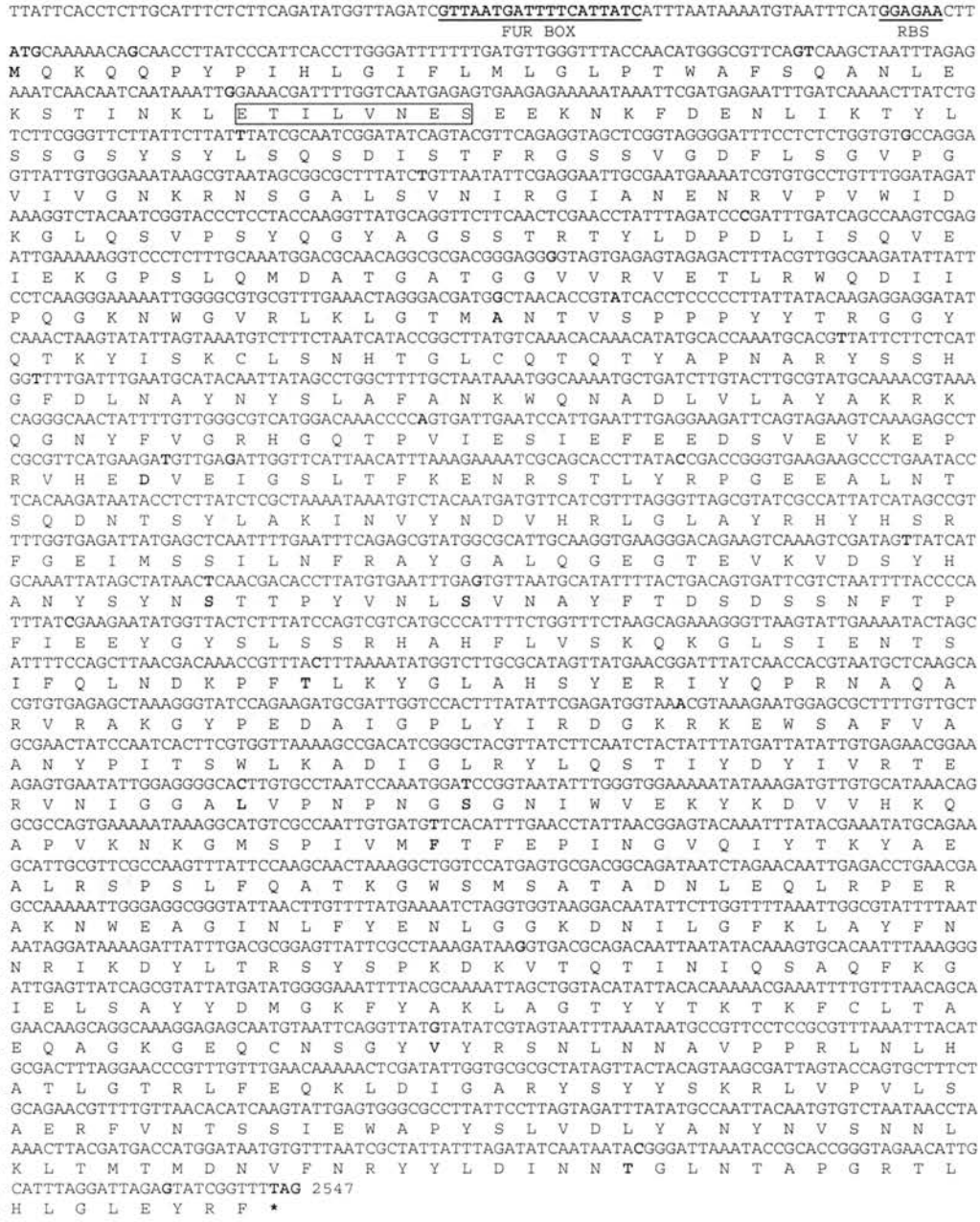

TTATTCACCTCTTGCATTTCTCTTCAGATATGGTTAGATCGTTAATGATTTTCATTATCATTTAATAAAATGTAATTTCATGGAGAACTT
FUR BOX
RBS
1 ATGCAAAAACAGCAACCTTATCCCATTCACCTTGGGATTTTTTTGATGTTGGGTTTACCAACATGGGCGTTCAGTCAAGCTAATTTAGAG
1 M Q K Q Q P Y P I H L G I F L M L G L P T W A F S Q A N L E
91 AAATCAACAATCAATAAATTGGAAACGATTTTGGTCAATGAGAGTGAAGAGAAAAATAAATTCGATGAGAATTTGATCAAAACTTATCTG
31 K S T I N K L E T I L V N E S E E K N K F D E N L I K T Y L
181 TCTTCGGGTTCTTATTCTTATTTATCGCAATCGGATATCAGTACGTTCAGAGGTAGCTCGGTAGGGGATTTCCTCTCTGGTGTGCCAGGA
61 S S G S Y S Y L S Q S D I S T F R G S S V G D F L S G V P G
271 GTTATTGTGGGAAATAAGCGTAATAGCGGCGCTTTATCTGTTAATATTCGAGGAATTGCGAATGAAAATCGTGTGCCTGTTTGGATAGAT
91 V I V G N K R N S G A L S V N I R G I A N E N R V P V W I D
361 AAAGGTCTACAATCGGTACCCTCCTACCAAGGTTATGCAGGTTCTTCAACTCGAACCTATTTAGATCCCGATTTGATCAGCCAAGTCGAG
121 K G L Q S V P S Y Q G Y A G S S T R T Y L D P D L I S Q V E
451 ATTGAAAAAGGTCCCTCTTTGCAAATGGACGCAACAGGCGCGACGGGAGGGGGTAGTGAGAGTAGAGACTTTACGTTGGCAAGATATTATT
151 I E K G P S L Q M D A T G A T G G V V R V E T L R W Q D I I
541 CCTCAAGGGAAAAATTGGGGCGTGCGTTTGAAACTAGGGACGATGGCTAACACCGTATCACCTCCCCCTTATTATACAAGAGGAGGATAT
181 P Q G K N W G V R L K L G T M A N T V S P P P Y Y T R G G Y
631 CAAACTAAGTATATTAGTAAATGTCTTTCTAATCATACCGGCTTATGTCAAACACAAACATATGCACCAAATGCACGTTATTCTTCTCAT
211 Q T K Y I S K C L S N H T G L C Q T Q T Y A P N A R Y S S H
721 GGTTTTGATTTGAATGCATACAATTATAGCCTGGCTTTTGCTAATAAATGGCAAAATGCTGATCTTGTACTTGCGTATGCAAAACGTAAA
241 G F D L N A Y N Y S L A F A N K W Q N A D L V L A Y A K R K
811 CAGGGCAACTATTTTGTTGGGCGTCATGGACAAACCCCAGTGATTGAATCCATTGAATTTGAGGAAGATTCAGTAGAAGTCAAAGAGCCT
271 Q G N Y F V G R H G Q T P V I E S I E F E E D S V E V K E P
901 CGCGTTCATGAAGATGTTGAGATTGGTTCATTAACATTTAAAGAAAATCGCAGCACCTTATACCGACCGGGTGAAGAAGCCCTGAATACC
301 R V H E D V E I G S L T F K E N R S T L Y R P G E E A L N T
991 TCACAAGATAATACCTCTTATCTCGCTAAAATAAATGTCTACAATGATGTTCATCGTTTAGGGTTAGCGTATCGCCATTATCATAGCCGT
331 S Q D N T S Y L A K I N V Y N D V H R L G L A Y R H Y H S R
1081 TTTGGTGAGATTATGAGCTCAATTTTGAATTTCAGAGCGTATGGCGCATTGCAAGGTGAAGGGACAGAAGTCAAAGTCGATAGTTATCAT
361 F G E I M S S I L N F R A Y G A L Q G E G T E V K V D S Y H
1171 GCAAATTATAGCTATAACTCAACGACACCTTATGTGAATTTGAGTGTTAATGCATATTTTACTGACAGTGATTCGTCTAATTTTACCCCA
391 A N Y S Y N S T T P Y V N L S V N A Y F T D S D S S N F T P
1261 TTTATCGAAGAATATGGTTACTCTTTATCCAGTCGTCATGCCCATTTTCTGGTTTCTAAGCAGAAAGGGTTAAGTATTGAAAATACTAGC
421 F I E E Y G Y S L S S R H A H F L V S K Q K G L S I E N T S
1351 ATTTTCCAGCTTAACGACAAACCGTTTACTTTAAAATATGGTCTTGCGCATAGTTATGAACGGATTTATCAACCACGTAATGCTCAAGCA
451 I F Q L N D K P F T L K Y G L A H S Y E R I Y Q P R N A Q A
1441 CGTGTGAGAGCTAAAGGGTATCCAGAAGATGCGATTGGTCCACTTTATATTCGAGATGGTAAACGTAAAGAATGGAGCGCTTTTGTTGCT
481 R V R A K G Y P E D A I G P L Y I R D G K R K E W S A F V A
1531 GCGAACTATCCAATCACTTCGTGGTTAAAAGCCGACATCGGGCTACGTTATCTTCAATCTACTATTTATGATTATATTGTGAGAACGGAA
511 A N Y P I T S W L K A D I G L R Y L Q S T I Y D Y I V R T E
1621 AGAGTGAATATTGGAGGGGCACTTGTGCCTAATCCAAATGGATCCGGTAATATTTGGGTGGAAAAATATAAAGATGTTGTGCATAAACAG
541 R V N I G G A L V P N P N G S G N I W V E K Y K D V V H K Q
1711 GCGCCAGTGAAAAATAAAGGCATGTCGCCAATTGTGATGTTCACATTTGAACCTATTAACGGAGTACAAATTTATACGAAATATGCAGAA
571 A P V K N K G M S P I V M F T F E P I N G V Q I Y T K Y A E
1801 GCATTGCGTTCGCCAAGTTTATTCCAAGCAACTAAAGGCTGGTCCATGAGTGCGACGGCAGATAATCTAGAACAATTGAGACCTGAACGA
601 A L R S P S L F Q A T K G W S M S A T A D N L E Q L R P E R
1891 GCCAAAAATTGGGAGGCGGGTATTAACTTGTTTTATGAAAATCTAGGTGGTAAGGACAATATTCTTGGTTTTAAATTGGCGTATTTTAAT
631 A K N W E A G I N L F Y E N L G G K D N I L G F K L A Y F N
1981 AATAGGATAAAAGATTATTTGACGCGGAGTTATTCGCCTAAAGATAAGGTGACGCAGACAATTAATATACAAAGTGCACAATTTAAAGGG
661 N R I K D Y L T R S Y S P K D K V T Q T I N I Q S A Q F K G
2071 ATTGAGTTATCAGCGTATTATGATATGGGGAAATTTTACGCAAAATTAGCTGGTACATATTACACAAAAACGAAATTTTGTTTAACAGCA
691 I E L S A Y Y D M G K F Y A K L A G T Y Y T K T K F C L T A
2161 GAACAAGCAGGCAAAGGAGAGCAATGTAATTCAGGTTATGTATATCGTAGTAATTTAAATAATGCCGTTCCTCCGCGTTTAAATTTACAT
721 E Q A G K G E Q C N S G Y V Y R S N L N N A V P P R L N L H
2251 GCGACTTTAGGAACCCGTTTGTTTGAACAAAAACTCGATATTGGTGCGCGCTATAGTTACTACAGTAAGCGATTAGTACCAGTGCTTTCT
751 A T L G T R L F E Q K L D I G A R Y S Y Y S K R L V P V L S
2341 GCAGAACGTTTTGTTAACACATCAAGTATTGAGTGGGCGCCTTATTCCTTAGTAGATTTATATGCCAATTACAATGTGTCTAATAACCTA
781 A E R F V N T S S I E W A P Y S L V D L Y A N Y N V S N N L
2431 AAACTTACGATGACCATGGATAATGTGTTTAATCGCTATTATTTAGATATCAATAATACGGGATTAAATACCGCACCGGGTAGAACATTG
811 K L T M T M D N V F N R Y Y L D I N N T G L N T A P G R T L
2521 CATTTAGGATTAGAGTATCGGTTTTAG 2547
841 H L G L E Y R F *

Fig. 11. Different protein prediction profiles of the deduced amino acid sequence of *P. multocida* HasR depicting antigenicity, hydrophylicity, surface probability and transmembrane plots. (A) Antigenicity plot of the deduced amino acid sequence of *P. multocida* A:3 HasR. The plot was generated using the Parker antigenicity algorithm in MacVector with a window size of 11 residues. (B) Hydrophilicity plot of HasR. The hydrophobic signal peptide is indicated. Positive values represent hydrophilic regions. The plot was generated using the Kyte-Doolittle algorithm in MacVector, with a window size of 7. Positive values represent hydrophilic regions. (C) Surface probability plot. (D) Von Heijne Transmembrane plot.

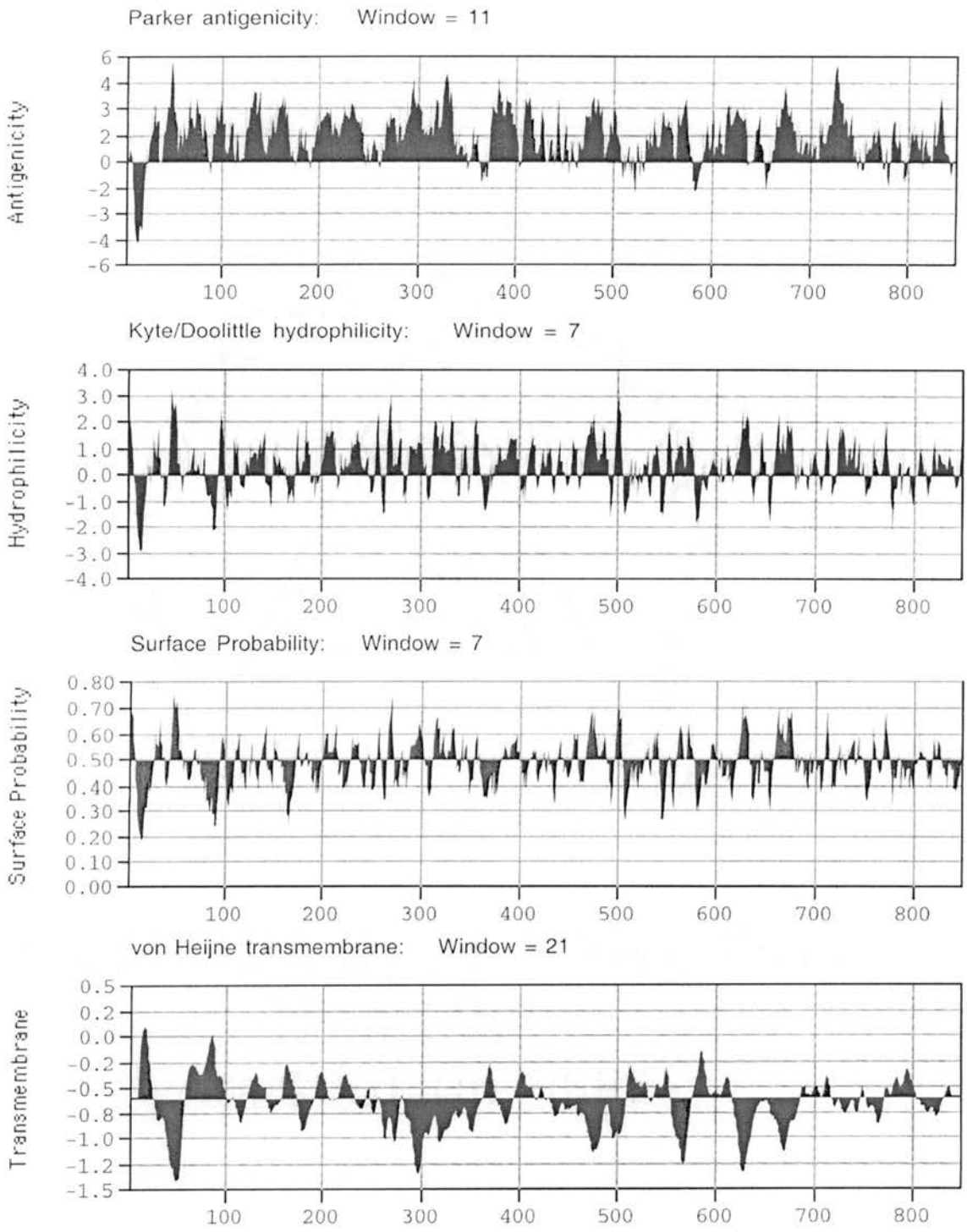

Parker antigenicity: Window = 11
Antigenicity
6
4
3
2
0
-2
-3
-4
-6
100
200
300
400
500
600
700
800
Kyte/Doolittle hydrophilicity: Window = 7
Hydrophilicity
4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
100
200
300
400
500
600
700
800
Surface Probability: Window = 7
Surface Probability
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
100
200
300
400
500
600
700
800
von Heijne transmembrane: Window = 21
Transmembrane
0.5
0.2
0.0
-0.2
-0.5
-0.8
-1.0
-1.2
-1.5
100
200
300
400
500
600
700
800

Fig. 12. Autoradiography of hemin-blot depicting hemin-binding proteins of *P. multocida* A:3. Lane 1- Heat-treated (100°C) whole cell lysates expressing IROMPs; lane 2 - non-heat treated (37°C) whole cell lysate; lane 3 – heat-treated IROMP-enriched fractions and lane 4 – non-heat treated IROMP-enriched fractions. This autoradiography clearly demonstrates that *P. multocida* A:3 has several hemin-binding proteins including HasR (arrow). The 96 kDa was not heat modifiable.

Hemin-binding blot

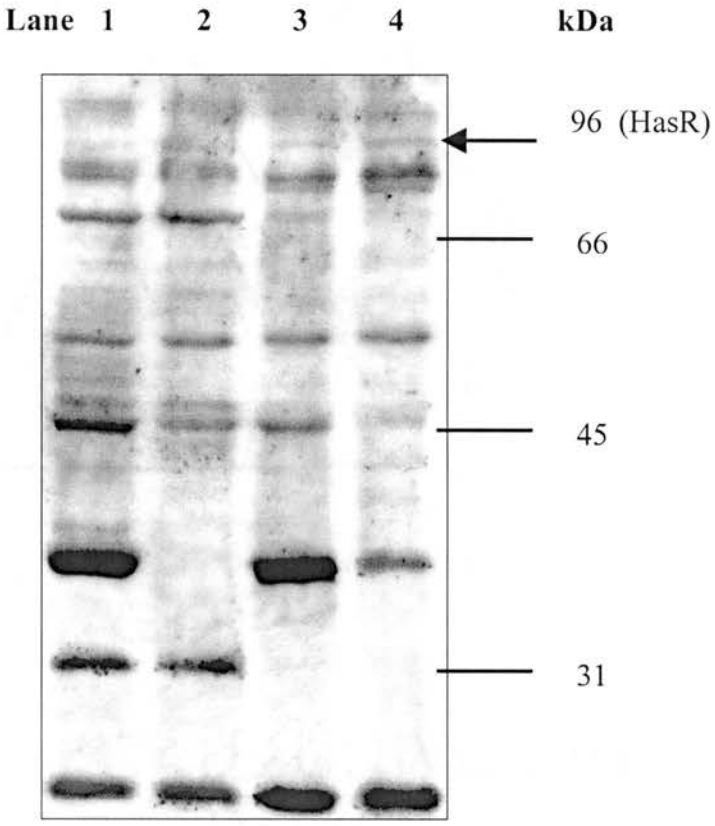


Fig. 13. Western immunoblot depicting surface antigens of *P. multocida* A:3 Pm232. Lane 1 – Intact rabbit serum, lane 2 – adsorbed rabbit serum on *P. multocida* and lane 3 – eluted rabbit serum antibodies from *P. multocida* surface antigens.

Western blot on *P. multocida* whole cells

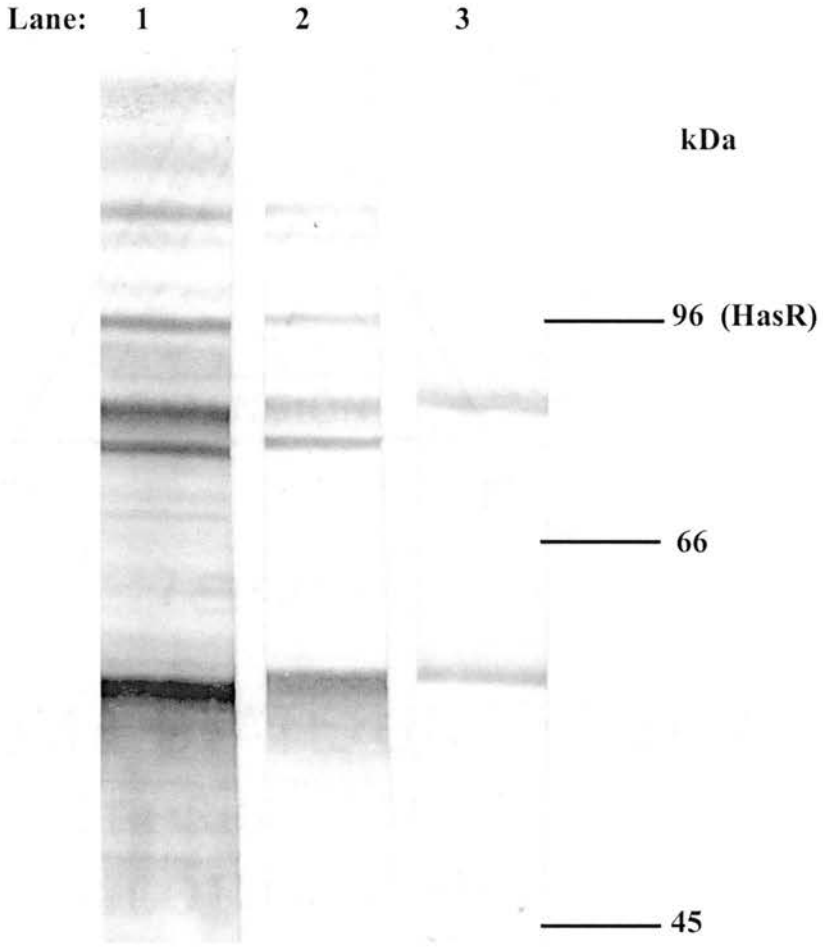
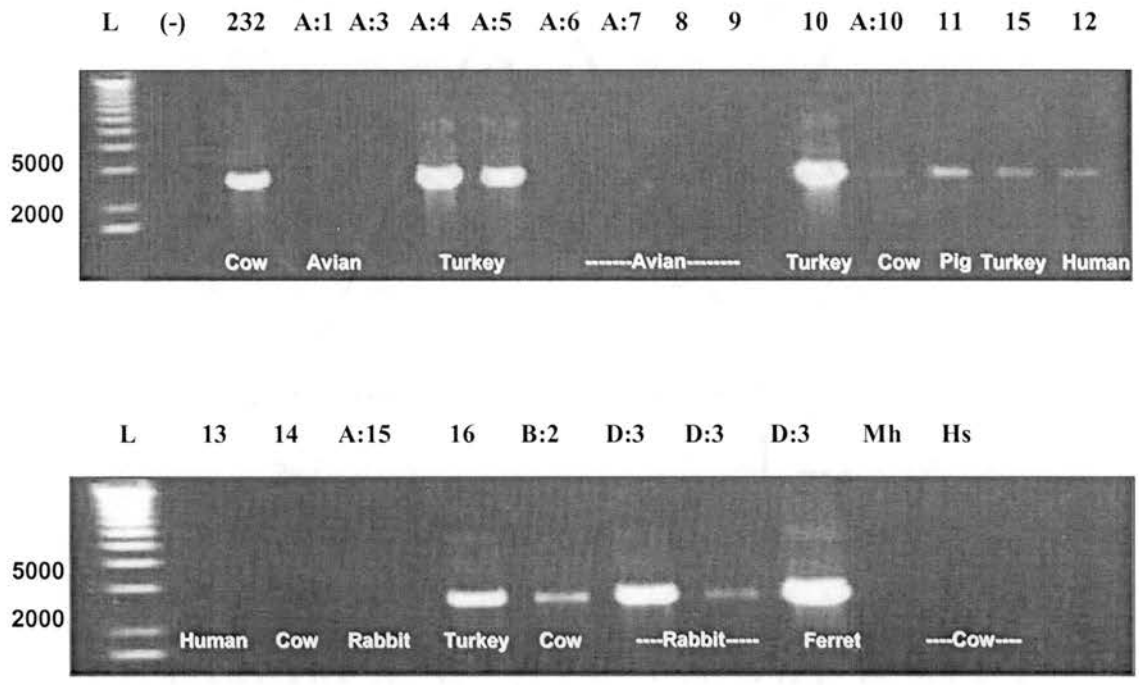


Fig. 14. Agarose gel showing results of amplification of the *hasR* ORF by PCR from genomic DNA of different *P. multocida* isolates. Gel #1: Lane 1 – 1 Kb plus ladder; lane 2 – Negative control; lane 3 – *P. multocida* A:3; lane 4 – A:1; lane 5 – A:3; lane 6 – A:4; lane 7 – A:5; lane 8 – A:6; lane 9 – A:7; lane 10 – 8; lane 11 – 9; lane 12 – 10; lane 13 – A:10; lane 14 – 11; lane 14 – 11; lane 15 – 15 and lane 16 – 12. Gel #2: Lane 1 – 1 Kb plus ladder; lane 2 –13; lane 3 – 14; lane 4 – A:15; lane 5 – 16; lane 6 – B:2; lane 7 – D:3; lane 8 – D:3; lane 9 – D:3; lane 10 – *M. haemolytica*; and lane 11 – *H. somnus*.


L (-) 232 A:1 A:3 A:4 A:5 A:6 A:7 8 9 10 A:10 11 15 12
5000
2000
Cow Avian Turkey ------Avian------- Turkey Cow Pig Turkey Human
L 13 14 A:15 16 B:2 D:3 D:3 D:3 Mh Hs
5000
2000
Human Cow Rabbit Turkey Cow ----Rabbit----- Ferret ----Cow----

VITA

Maria Eugenia Prado

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Doctor of Philosophy

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