MANNHEIMIA HAEMOLYTICA-INDUCED CYTOKINE GENE MODULATION IN BOVINE LEUKOCYTES

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2002

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2008

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TABLE OF CONTENTS

CHAPTER I
INTRODUCTION

Mannheimia haemolytica is the major cause of fibrinous and necrotizing pleuropneumonia of cattle termed bovine pneumonic pasteurellosis (BPP) or shipping fever. BPP has a significant economic impact costing approximately $ 3 billion annually to the US cattle industry alone. Several virulence factors of *M. haemolytica* promote host-pathogen interactions by assisting the bacterium to colonize cattle lungs and contribute to the development of pneumonia. Leukotoxin (LKT) and lipopolysaccharide (LPS) are two critically important virulence factors, and their roles in the pathogenesis of shipping fever are well documented. Other virulence factors with less documented pathogenic roles include capsule, fimbriae, iron-regulated proteins, outer membrane proteins, neuraminidase, and a neutral glycoprotease.

Most of the knowledge that has been generated about the role of LKT and LPS in the pathogenesis of shipping fever comes from multiple *in vitro* studies using purified LKT alone or in combination with LPS. *In vivo* studies using whole bacteria are limited. Studies using whole bacteria containing all virulence factors should provide better insight into the pathogenesis of shipping fever. Therefore, we investigated the role of LKT by challenging bovine alveolar macrophages (BAM) and live calves with LKT-deficient and wild type *M. haemolytica* strains.

The hypothesis was that cytokine mRNA modulation in inflammatory cells is influenced by LKT. The experiments were designed in two parts, *in vitro* followed by an *in vivo* experiment. For *in vitro* experiment, BAM were challenged with the wild type and LKT-deficient *M. haemolytica* at the concentration of 1 bacterium per BAM. The effects on macrophage function were measured by quantifying the cytokine response of TNFα, IL-1ß, IL-8, IL-10 and IL-12 using ELISA and real-time RT-PCR. *In vivo* experiment was

designed to further investigate the role of LKT in modulating cytokine gene and protein expression in bovine inflammatory cells obtained from the lungs of calves challenged by an isogenic LKT deficient and wt *M. haemolytica* strains.

A growing body of evidence implicates multiple inflammatory cytokines in the pathogenesis of BPP. Heat-killed *M. haemolytica*, as well as purified LPS and LKT induce the expression of cytokines TNFα, IL-1β, and IL-8 genes and proteins in bovine alveolar macrophages *in vitro*. We identified cytokines TNFα, IL-1ß, and IL-8 because these are pleiotropic early response proinflammatory molecules produced by a variety of cells and play significant roles in innate immunity. The cytokine IL-12 was selected for measurement because it is involved principally in regulating-adaptive immune response. The principal routine function of IL-10 appears to be to limit and ultimately terminate inflammatory responses.

CHAPTER II

REVIEW OF LITERATURE

Virulence factors associated with *Mannheimia haemolytica* infection in cattle

Table of contents

Abstract

Mannheimia haemolytica S1 is considered the predominant cause of bovine pneumonic pasteurellosis (BPP) or shipping fever. Various virulence factors allow *M. haemolytica* to colonize the lungs and establish infection; the foremost of these virulence factors is the leukotoxin (LKT). Other virulence factors include adhesin; capsule, outer membrane proteins, and various proteases used for evading innate and adaptive host immune responses. The effects of LKT are ruminant species-specific and follow a dose dependent activation-inhibition paradox on bovine leucocytes. At very low concentrations, LKT can activate bovine leukocytes to undergo respiratory burst and degranulation, stimulate cytokine release from macrophages, and stimulate histamine release from mast cells. At high concentration LKT stimulates cells to undergo apoptosis; while still at higher concentration, leukotoxin results into formation of transmembrane pores and subsequent cell necrosis. The species specificity of LKT stem from a unique interaction with the bovine β2 integrin receptor. The interaction of LKT with alveolar macrophages and neutrophils is followed by activation of these leukocytes to undergo oxidative burst and release of proinflammatory cytokines such as IL-1, IL-8, IL-6, and TNF alpha. Under the similar effects induced by LPS, inflammatory cells conglomerate in the lungs. Formation of transmembrane pores in activated leukocytes results in release of products reactive oxygen radicals, lysosomal enzymes, and arachidonic acid metabolites into pulmonary parenchyma resulting in tissue destruction and bronchopneumonia.

Introduction

M. haemolytica is the major cause of fibrinous and necrotizing bronchopneumonia of cattle termed bovine pneumonic pasteurellosis (BPP) or Shipping Fever. BPP has a marked economic significance worldwide and costs approximately $ 1 billion annually to the US cattle industry [1]. The term 'shipping fever' emphasizes circumstances (stress) under which the disease predominantly occurs. Various combinations of environmental stress such as inclement weather, shipment, weaning, overcrowding and complex interactions between several infectious agents including Bovine herpesvirus -1 (BHV-1), parainfluenza 3 virus (PI-3), bovine respiratory syncytial virus (BRSV), bovine viral diarrhea virus (BVDV) and bacteria can act as predisposing factors. Although, the shipping-fever is a multifactorial disease, *M. haemolytica* serotype S1 is considered the major cause of pneumonia and it is generally believed that control of *M. haemolytica* serotype S1 infection would markedly reduce the prevalence and severity of shipping fever.

M. haemolytica is an opportunistic pathogen; it is a normal inhabitant of nasopharynx and tonsils of cattle and sheep[2, 3]. In healthy cattle, the relatively nonpathogenic *M. haemolytica* serotypes S2 and S4 predominate, while the pathogenic serotype S1 is present in low numbers [4, 5]. Although the exact mechanisms are not known, stress results in microenvironment changes favorable for multiplication and colonization of serotype S1 in the upper respiratory tract. The selective growth and colonization of nasopharyngeal region by *M. haemolytica* serotype S1 is a prerequisite for the development of shipping fever [6]. Resulting colonization of nasal mucosa with large numbers of serotype S1 contributes to the inhalation of aerosol droplets containing bacteria into the trachea and lungs [7]. Healthy calves can clear the inhaled bacteria, whereas stressed calves develop pneumonia. Although the most common isolate from the cattle lung with shipping fever is *M. haemolytica* serotype S1, other reported serotypes include S2,S5, S6, S9[8, 9].

The underlying pathologic process by which various environmental factors alone or in combination with certain microorganisms predispose cattle to shipping fever is unknown. Presumably, alteration of local innate and adaptive immune responses contribute to the development of the disease process[10]. In experimental inoculation of calves with BVDV, there is a significant reduction in the numbers of CD4+ and CD8+ lymphocytes in the peripheral blood that can alter adaptive immune response [10, 11]. Calves infected with BHV-1 cannot clear inhaled bacteria due to the damaged mucociliary apparatus and altered alveolar macrophage functions [11]. Leite et al., (2005) suggested that incubation of bovine neutrophils with the medium from BHV-1-infected peripheral blood mononuclear cells increase their susceptibility to *M. haemolytica* exotoxin leukotoxin (LKT) by increasing expression of LKT receptors on neutrophils [12]. Likewise, the *in vitro* susceptibility of bighorn sheep neutrophils to LKT was increased by prior exposure to elevated plasma cortisol concentrations [13]. It is, therefore, possible that a similar process occurs in neutrophils and alveolar macrophages *in vivo* thereby predisposing calves to BPP [13]. Additionally, physical factors such as exposure of calves to cold air not only disrupted normal lung functions as shown by the altered ventilation and gas exchange, but also increased cortisol levels and altered neutrophil functions [14-16]. Alternatively, it is also possible that, stress-associated changes may modulate expression of bacterial surface proteins and enzymes thereby enhancing pulmonary colonization [1].

Various virulence factors possessed by *M. haemolytica* S1 promote lung colonization and evasion of host immune response. Therefore, the host immune status is critical in counteracting these strategies. If the host immune response is not strong enough to curtail the pulmonary colonization, these interactions contribute to the development of BPP. Multiple review articles address these interactions in detail[17, 18].Several virulence factors of *M. haemolytica* serotype S1 promote host-pathogen interaction and influence the outcome of these interactions. Out of these, LKT and lipopolysaccharide (LPS) are critically important virulence factors, and their roles in pathogenesis of shipping fever are well documented [6, 17, 19, 20]. Other virulence factors whose pathogenic roles are less well documented or not defined include capsule,

adhesin, outer membrane proteins, and various proteases such as neuraminidase, glycoprotease, and metalloglycoprotease [19, 19, 21-31]. Following is a brief overview of the virulence factors associated with *M. haemolytica* serotype S1.

1. Surface proteins and carbohydrates

a. Adhesins

A naïve host encounters *M. haemolytica* from the environment through air or droplet inhalation. A necessary requirement for development of BPP is initial colonization and establishment in nasopharynx by adherence to the epithelial surface [32]. This binding must be such that it provides resistance to physical removal by air flow and by local innate immune mechanisms such as mucosal clearance. The specific adhesins of *M. haemolytica* involved in nasopharyngeal epithelial binding are not fully understood [33]. De la Mora et al., (2006) demonstrated a 68 kDa adhesin molecule associated with *M. haemolytica,* which is involved in adhesion to tracheal cell cultures [22]. In addition to binding tracheal cell-cultures, this adhesin also bound a 165-kDa glycoprotein receptor on neutrophils and activated them to undergo an oxidative burst.[22] Whether this adhesin is involved in *in vivo* localization has yet to be established. Another, putative adhesin molecule that is present on fimbriae of *M. haemolytica* binds to a sialoglycoprotein receptor on respiratory epithelium[21]. However, a locus encoding such an adhesin has not yet been identified [33]. Alternatively, adhesion molecules are not always necessary for niche colonization, because of nonspecific adherence to epithelial surface via capsular or other bacterial surface proteins can occur or bacteria can simply remain within the mucus layer. This view is supported by the fact that adherence of *M. haemolytica* to nasal mucus can be altered by enzymatic degradation of protein and carbohydrate component of mucus [34]. *M. haemolytica*-derived neuraminidase has been shown to exert a pathogenic role by degrading mucus components enhancing *M. haemolytica* adherence to epithelium[31]. Retzer et al., (1998) proposed outer membrane iron-binding proteins known as TbpA and TbpB, which are involved in iron acquisition, to potentially serve as an adhesin molecule because of their homologue in *Neisseria meningitidis* is expressed on bacterial surface

[35]. Furthermore, based on evaluation of the *M. haemolytica* genome sequence comparision with a motif from *Neisseria meningitidis* and *Bordetella* sp. filamentous hemagglutinin, Highlander proposed another high molecular weight (340 Kd) filamentous hemagglutinin as an adhesin molecule [33]. Additionally, other putative adhesin-like sequences have been observed in the genome sequence of *M. haemolytica* S1; analysis of those proteins is awaited [33, 36].

b. Lipopolysaccharide

LPS is an important virulence factor and plays a critical role in the pathogenesis of BPP. Current evidence indicates that LPS contributes to pulmonary pathology through a variety of complex mechanisms including the stimulation of leukocytes to produce proinflammatory cytokines, activation of complement and coagulation cascade, and direct cell cytolysis[23, 23, 37]. LPS is a highly dynamic outer membrane component that can adapt to the surroundings. Davies et al., (1997 and 1996) demonstrated distinct serological difference between LPS molecules extracted from different serotypes of *M. haemolytica*[38-40]. The LPS molecule of most serotypes consists of O-antigen, lipid A, and inner and outer core of oligosaccharides giving rise to smooth colonies due to its ability to bind water, whereas Serotype S2 and S8 lack O-antigen and are rough[41, 42]. The lipid A moiety of LPS is responsible for eliciting endotoxic effects such as pyrexia and hypotensive shock. LPS is a potent vasodilator and when administered intravenously in sheep it produced clinical signs of hypotension (Adlam, 1989). The systemic effects of LPS are considerably important in acute pasteurellosis, which causes septicemia in lambs resulting in high mortality. The pathological effects of LPS are mediated by binding of LPS with LPS-binding protein (LBP) and subsequently further interactions are, most-likely, mediated by CD 14. LPS forms high molecular weight complexes with LKT thus working in synergy by promoting the pathogenic-effect of each other[23, 43]. Additionally, *in vitro* studies revealed that bovine alveolar macrophages (BAM) challenged simultaneously with both LKT and LPS produced more TNF alpha and IL-8 compared to cells challenged with each factor individually[23].

In the pneumonic calf lungs, LPS can rapidly cross the alveolar wall and was found localized within the cytoplasm of neutrophils, alveolar macrophages, endothelial cells, pulmonary intravascular macrophages, and on epithelial cell surfaces [44]. It can stimulate alveolar macrophages to produce proinflammatory cytokines, reactive nitrogen intermediates, reactive oxygen intermediates, and other mediators that can participate actively in the inflammatory process [45, 46]. Subsequently, these proinflammatory cytokines and chemotactic mediators initiate influx of neutrophils [23, 47]. *In vitro* challenge of bovine neutrophils with both LPS and LKT resulted in degranulation, generation of superoxide, and nitric oxide and lysis[48]. Another *in vitro* study demonstrated increased expression of elastase, myeloperoxidase (MPO), nitric oxide and alkaline phosphatase in neutrophils [49]. The *in vitro* effect of LPS on BAMs demonstrated a dose dependent increase in iNOS gene expression, and NO generated from LPS-stimulated BAMs caused cytotoxic injury to pulmonary endothelial cells in a dose-dependent manner [46]. Inculcation of *M. haemolytica* in calf lung lobes revealed iNOS gene expression in leukocytes and epithelial cells [50].

LPS can cause pulmonary damage directly through toxic effects on pulmonary endothelium and indirectly through neutrophil recruitment [51, 52]. The toxicity of LPS can be enhanced by complexing it with the phospholipids in the pulmonary surfactant which can help in pulmonary colonization by evading local innate immunity, thereby, allowing it to persist in the lung and initiate inflammation [53, 54]. Unfortunately, LPS used in this study was not derived from *M. haemolytica* and similar study was not conducted with surfactant obtained from calves with BPP; therefore, whether LPS of *M. haemolytica* uses similar mechanism is not known. The interaction of LPS with infiltrating leukocytes stimulates production of various proinflammatory cytokines e.g. IL-1 beta, IL-8, LTB4, prostaglandin E2, and TNF alpha from bovine leukocytes[23, 37, 51]. Other systemic effects of LPS include fever and production of acute phase proteins by liver.

Similar to LKT, LPS exhibits a dose dependent effect on the bovine peripheral blood leukocytes. At low concentrations, LPS decreased the phagocytic ability of

neutrophils, while at higher concentrations the phagocytic activity was increased (ref). Moderate concentration was mitogenic for mononuclear cells, whereas a high concentration had the opposite effects [55]. The effects of purified *M. haemolytica* LPS infusion at the cellular level were studied by Emau et al., 1987[56]. The authors concluded that the action of LPS at the cellular level was mediated by cyclic nucleotides as evidenced by an increase in plasma arachidonic acid, thromboxane B2 and prostaglandin E levels[56]. Although, O-antigen of LPS is immunogenic and antibodies exhibit cross-reactivity among different serotypes, there is no correlation between high antibody response to LPS and protection of the host from development of pneumonia [19, 57].

c. Capsule:

There are 12 serotypes of *M. haemolytica* (S1, S2, S5-S9, S12-14, S16 and S17) based on the differences in capsular polysaccharide antigen typing. Log-phase bacteria exhibit good encapsulation, whereas stationary-phase bacteria exhibit poor encapsulation [58]. Several authors have shown similarities in between the capsular polysaccharides of *M. haemolytica* and other bacterial capsular polysaccharides; for instance, S2 capsular polymer is identical with capsular polysaccharides of *Neisseria meningitidis* B and *E. coli* K1 suggesting evolutionary relationship [59, 60].

The capsule of *M. haemolytica* S1 may be involved in colonization of lung by promoting bacterial adherence to respiratory epithelium [61]. Except for lung colonization, the specific role of capsule in the pathogenesis is not yet established. Several studies demonstrated that it may inhibit phagocytosis by neutrophils and macrophage and complement-mediated lysis of bacteria [62-64] whereas others found that the presence of capsule promoted phagocytosis and subsequent killing [65]. Vaccination with capsular antigens promote production of anti-capsular antibodies, a significant positive correlation was not established between the capsular antibody and protection [25].

d. Outer membrane proteins

Mannheimia haemolytica possess multiple outer membrane proteins (OMP)[66]. Several of these proteins are iron-regulated outer membrane proteins (IROMPs) and are physiologically and pathologically relevant because they are involved in iron acquisition such as transferrin binding protein 1 and 2 (Tbp 1 and Tbp 2); also called as TbpA and TbpB [67]. Because *M. haemolytica* does not produce siderophores, expression of these IROMPs is considered to be the main iron acquisition mechanism of *M. haemolytica*[67, 68]. Iovane et al., (1998) demonstrated that OMP can also act as chemotactic agents for,neutrophils and inhibit their phagocytic and subsequent bacterial killing mechanisms thereby favoring bacterial pulmonary colonization[69]. These authors used multiple OMPs (28-40 kDa) and did not specify the strain of bacteria used for collecting the proteins. Detailed understanding of the protective antigens of *M. haemolytica* is not completely understood, but data indicate that the protective immunity against *M. haemolytica* can be acquired through the production of neutralizing antibodies to LKT and antibodies that bind to outer membrane proteins (OMP)[30, 70]. Pandher et al. (1998) identified 21 surface-exposed immunogenic OMPs from *M. haemolytica* S1[71]. One of the immunogenic OMPs that offers protection following vaccination is a 45 kDa serotype S1 outer membrane lipoprotein PlpE[66, 71]. The antibodies generated against PlpE offer cross protection against serotype 6 and promote phagocytosis and complement mediated killing of bacteria[71]. Another outer membrane protein, OmpA, is a highly conserved protein that acts as a ligand and is involved in binding to specific host cell receptors in the upper respiratory tract thereby playing role in adherence and colonization and in generating host-specificity[72]. In addition, outer membrane porins from *M haemolytica* stimulated the release of NO, the expression of iNOS in IFNγ activated macrophages, increased actin polymerization, and modified oxidative burst in neutrophils [69, 73].

3. Toxins and extracellular enzymes

a. Leukotoxin

M. haemolytica LKT and LPS are the best characterized virulence factors with respect to their pathogenic role in the shipping fever [33, 74]. Since its initial discovery, LKT has been the subject of intense investigation relative to its role in the pathogenesis

of shipping fever. Leukotoxin is an exotoxin and a member of the RTX (Repeats in Toxin) family of toxins. These toxins are genetically related and share a common highly conserved motif consisting of a series of glycine-aspartic acid nonapeptide repeats in the carboxy terminal third of the LKT protein molecule. This conserved motif is involved in calcium binding and plays a vital role in inducing leukocytic toxicity due to its ability to form tertiary conformation required for target host cell binding [75]. The conserved motif also contains a recognition site required for transport of LKT across biological membranes in bacteria by the *tolC*-dependent type I secretion system [76, 77]. The other members of this family and their leukotoxins include *Actinobacillus actinomycetemcomitans* (LtxA), *Actinobacillus pleuropneumoniae* cytotoxins (ApxIA, ApxIIA, and ApxIIIA), *E. coli* alpha hemolysin, and *Bordetella pertussis* hemolysin. Although these members belonging to RTX family of toxins are genetically related due to the similarity of mechanisms involved in LKT activation, secretion and partially shared amino acid sequences, they differ markedly in target cell specificity[78].

M. haemolytica LKT is a 102-105 kDa protein, produced by all serotypes during the logarithmic phase of bacterial growth *in vitro* [79]. The genetic organization of the LKT gene complex is composed of four genes with genes *lktC* and gene *lktA* located upstream and genes *lktB* and *lktD* located downstream[80]. The structural gene product of *lktA* is composed of 953 amino acids and encodes the biologically inactive component of LKT - protoxin (pro-LKT-A). Gene *lktC* encodes transacylase that post-translationally modifies the inactive pro-LKT-A by fatty acid acylation thereby converting it into a biologically active leukotoxin (LKT-A). During acylation, the fatty acid groups are added to the lysine residues located on LKT-A, which is a critical step in removing charge and increasing the hydrophobicity of LKT-A thereby providing biochemical alternation allowing LKT-A to insert in host cells. Although acylation is not required for LKT-A binding to the target cells, it is required for elevation of intracellular calcium ion concentration, generation of reactive oxygen species, production of IL-8 and causing cytolysis[81]. Gene products of *lktB* and *lktD* are required for transport of LKT-A from the bacterial cytoplasm into the outer environment (Fig. 1).

LKT-A contains domains that are responsible for receptor binding, pore-formation and calcium binding[75, 82]. The N-terminal region is putatively involved in the receptor binding, whereas the adjacent residues of LKT-A consist of a series of hydrophobic residues that are implicated in spanning the host cell membrane and possibly pore formation[83]. The carboxy terminal domain of LKT-A contains glycine and aspartate-rich repeats of RTX toxins. Most neutralizing epitopes are present within a 229 amino acid region at the C-terminal end of LKT-A[80].

LKT follows a species-specific, dose-dependent, activation-inhibition paradox on bovine leukocytes [84]. At low concentrations, LKT can activate neutrophils and macrophages to stimulate the respiratory burst and degranulation, stimulate proinflammatory cytokine (TNFα, IL-1 and IL-8) release from neutrophils and alveolar macrophages, reduce mitogen-mediated lymphoid proliferation, and stimulate histamine release from mast cells[48, 51]. At high concentrations, LKT stimulates bovine leukocytes to undergo apoptosis by engaging both extrinsic and intrinsic mechanisms, whereas at highest concentrations, LKT causes transmembrane pore formation, cell swelling and subsequent cell necrosis (oncotic cell death) [85-87]. The transmembrane pores in the plasma membrane of activated macrophages and neutrophils cause leakage of products of respiratory burst i.e. oxygen free radicals, superoxide anions, and hydrogen peroxide and other products such as nitric oxide, lysosomal enzymes including myeloperoxidase; arachidonic acid metabolites such as leukotriene B4 and 5-hydroxyeicosatetraenoic acid into the surrounding pulmonary parenchyma [46, 48, 62, 88].

LKT-A is a key virulence factor that contributes to the pathogenesis of the lung injury in BPP and is specific for ruminant leukocytes[29, 89]. In diseased lungs, LKT is associated with the cell membranes of degenerating inflammatory cells located in alveoli[44]. Although most strains of *M. haemolytica* from cattle and sheep produce leukotoxin, not all strains are equally pathogenic because LKT from these strains exhibit heterogeneity in terms of leukotoxic activity and amount of LKT produced, also there is a diversity of LKT structural gene *lktA* among strains [90, 91]. The importance of LKT in

causing pulmonary damage was established by intratracheal inoculation of calves with a LKT-deficient mutant *M. haemolytica*, which caused lower mortality and decreased lung lesions compared to animals challenged with the parent strain of bacterium that was capable of producing biologically active LKT [29, 89].

M. haemolytica induced pneumonia and other diseases are observed only in ruminants including cattle, sheep, bighorn sheep, goats, bison, and exotic ruminants because LKT-induced effects are specific for ruminant leukocytes including macrophages, lymphocytes, platelets, and neutrophils [92, 93]. This unique species-specificity stems from the selective interaction of LKT with the β_2 integrin receptor on target host cells [92, 94[2]]. Integrins are expressed on all cell types and are involved in cell-to-cell and cell-to-extracellular matrix interactions, whereas subtype β_2 integrins are expressed exclusively on leukocytes including T lymphocytes, macrophages, neutrophils, monocytes and dendritic cells[95]. In cattle and sheep, β_2 integrins have been identified as the receptors for LKT binding and therefore important for its subsequent effects[94, 96-99]. Although there is a consensus that LKT-induced specificity is due to β_2 integrins binding, a complete agreement is lacking on specific β2 integrins and subunit of β2 integrins required for receptor-ligand interaction[92, 94, 100]. Most of the scientific evidence support LKT interaction with the CD18 subunit of LFA-1 β_2 integrin[93]. Furthermore, *in vitro* incubation of bovine leukocytes with proinflammatory cytokines (IL-1β and TNFά) resulted in increased expression of LFA-1 and a simultaneously increase LKT binding, cytotoxicity, and apoptosis[12, 101, 102]. Similarly, *in vivo* and *in vitro* results indicate that bovine herpesvirus-1 infection increases susceptibility of the bovine leukocytes to LKT binding and cytotoxicity by increasing LFA-1 on neutrophils and peripheral blood mononuclear cells [103]. Recent studies using LKT-resistant mouse histiocytoma, human K562, and other cell lines demonstrated that these cell lines could be rendered susceptible of LKT effects by expressing cattle CD18 subunits on these cells. However, other evidence suggests LKT binds not only with the CD18 subunit of other β_2 integrins but also with the CD-11subunit [86, 98]. It appears that binding of LKT to CD-18 is critical in eliciting the pathologic effects of LKT, and during this binding LKT may interact with CD11 subunit [92, 93, 104]. Recently, it was suggested that LKT binds, in

particular, to the integrin epidermal growth factor 3 (I-EGF)-like domain of bovine CD18 [105]. It has also been suggested that non-β2 integrins-mediated interaction may also occur not only with cells from non ruminant species but also with cells lacking β2 integrins such as erythrocytes and platelets [82]. It is therefore possible that some effects are mediated by non-specific receptor ligand interactions.

In addition, limited *in vitro* studies reveal that LKT at sublethal dose is capable of inhibiting concanavalin, pokeweed mitogen, and purified protein derivative induced lymphoid proliferation which was partially abrogated by preincubation of lymphocytes with IL- 1 and IL- 2. The exact mechanism underlying this response is unknown; however, authors suggested failure of IL-2 production by lymphocytes or altered IL- 2 receptor expression [106, 107]. This affect raises interesting concern on whether increased susceptibility to other bacteria or virus in BPP is due to the underlying lymphoid necrosis. However, lymphopenia is not a clinical feature of BPP and LKT is only restricted to lungs of affected animal with no systemic distribution [44] suggesting that LKT induced inhibition of lymphoid proliferation may only be an *in vitro* phenomenon.

LKT Toxic mechanism

LKT-induced cytotoxicity of host leukocytes is characterized by the formation of transmembrane pores and eventual cell lysis [108, 109]. It is known, that LKT inserts its N-terminus into the host cell membrane to form a hydrophilic pore, but how these terminal transmembrane pores are formed is controversial. Studies indicate polymerization of LKT occurs on host cells, thereby forming transmembrane pores, whereas others suggest LKT-induced regulation of voltage gated channels and thereafter formation of transmembrane pores[108, 110, 111]. Irrespective of what basic process is involved in pore formation, these pores are 0.001-0.002 μm in diameter, allow rapid loss of intracellular K^+ from the host cell cytoplasm, and internalization of Na^+ molecules causing colloid-osmotic imbalances[108]. Subsequently water moves into the cell to correct the colloid-osmotic imbalance resulting in rapid cell swelling[109]. Uncontrolled

transmembrane calcium influx occurs and likely activates membrane phospholipases and proteases or may disrupt the cytoskeletal structure. The outcome of these alterations is degradation of the cell membrane and subsequent cytolysis of leukocytes as demonstrated by release of cytoplasmic lactate dehydrogenase [109]. Similarly, LKT can enhance ruminant platelet adhesion and activate and induce transmembrane pore formation in platelets by calcium dependent mechanisms [112-114]. The transmembrane pore in the plasma membrane of activated macrophages and neutrophils cause leakage of products of respiratory burst, lysosomal enzymes, arachidonic acid metabolites into the pulmonary parenchyma [46, 48, 88, 115]. These byproducts inadvertently cause damage to the pulmonary parenchyma giving rise to fibrinous and necrotizing bronchopneumonia typical of BPP [88]. LKT can also induce release of histamine from isolated bovine mast cells which results into rapid influx of serum proteins and more neutrophils at the location of inflammation; thereby, increasing the population of susceptible cells in the lung[116]. Because β2 integrins are present on lymphocytes as well, LKT induced damage to these cells allow bacteria to evade host adaptive immune responses.

The influence of calcium ions on the cytotoxic activity of *M. haemolytica* LKT is critical, because depletion of intracellular calcium-ion by addition of a calcium chelator eliminated the cytolytic effect of low doses of the toxin. Likewise, addition of calcium to target bovine leukemia cell line (BL-3) cell cultures depleted of the divalent cation restored the cytolytic effect of the leukotoxin. Addition of a calcium channel blocker resulted in dose-dependent protection BL-3. These results suggest that calcium positively influences the rapid initial phase of cell death resulting from exposure to the toxin [51, 117].

b. Proteases

A number of proteases have been associated with *M. haemolytica*. Although their specific physiological and pathological roles are not completely understood, some of them have been shown to be involved in circumventing innate and adaptive immune

response thereby enhancing lung colonization and establishment of the disease. In the bovine respiratory tract, IgA predominates in the upper respiratory tract whereas IgG is primary antibody in lower respiratory tract [118, 119]. Both IgG1 and IgG2 are believed to be important in defense against *M. haemolytica* infection. Glycoprotease obtained from the culture supernatant of *M. haemolytica* can selectively hydrolyze IgG1 thereby reducing opsonization induced phagocytosis and killing of bacteria [120]. Furthermore vaccination of calves with the recombinant glycoprotease alone and in combination with LKT provide protection against the disease [121]. All serotypes of *M. haemolytica* produce a zinc metalloglycoprotease (sialoglycoprotease) which can selectively degrade o-sialoglycoproteins [28, 122]. The precise pathogenic functions of this enzyme are not known; it is thought to involve adherence of bacteria to host epithelial cells; aggregation of platelets in the alveoli, and its activity can be potentiated by co-incubation with the LKT [113]. All strains of *M. haemolytica* produce another proteolytic enzyme neuraminidase, the precise function of which is also not known [31, 123]. Many respiratory pathogens, including *Hemophilus influenzae*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa*, express neuraminidases (sialidases) that can cleave α2,3-linked sialic acids from glycoconjugates. As mucosal surfaces are heavily sialylated, neuraminidases have been thought to modify epithelial cells by exposing potential bacterial receptors; enhance biofilm formation; and evading local innate immune response by cleaving salivary glycoproteins [124-126]. Therefore, it is possible that neuraminidase produced by *M. haemolytica* may play a role in initial colonization and evasion of antimicrobial killing and immune response by forming biofilm.

Summary

The pathogenesis of BPP is elaborate and complicated. In summary, *M. haemolytica* S1 is a normal inhabitant of upper respiratory tract of cattle. Predisposing factors include stress induced either by change in the environment (shipment, inclement weather, cold air) or by microbial agents (BHV-1, PI-3, BRSV, and BVDV infection). In the alveolar sacs, it initially interacts with the resident pulmonary alveolar macrophages that serve as primary line of host-defense. By various virulence factors, *M. haemolytica* can evade the immune response, colonize lungs and establish the infection. The foremost

of these virulence factors are LKT and LPS. In low sub cytotoxic dose LKT can activate macrophages through its interaction with its receptor ß2 integrins which is present not only on macrophages but also on other ruminant leukocytes including neutrophils and lymphocytes[48, 73]. This interaction of LKT with bovine leukocytes is followed by activation of leukocytes to undergo oxidative burst and release proinflammatory cytokines such as IL-1, IL-8, and TNFα (Fig. 2)[37, 53]. Under the similar effects induced by LPS, inflammatory cells conglomerate in the lungs[23, 45]. To overcome host defenses, *M. haemolytica* has evolved a powerful mechanism of inducing LKT mediated cytotoxicity of the leukocytes involved in the innate and adaptive immune response [109]. LKT forms transmembrane pores in bovine lymphocytes, neutrophils, macrophages, and mast cells and eventually resulting in cell cytotoxicity (Fig. 2)[109, 127, 128].These transmembrane pore in the plasma membrane of activated macrophages and neutrophils cause leakage of products of respiratory burst, lysosomal enzymes, arachidonic acid metabolites such as leukotriene B4 and 5-hydroxyeicosatetraenoic acid into the pulmonary parenchyma[48, 88, 101].These byproducts inadvertently damage the pulmonary parenchyma[88]. Moreover, increased expression of LKT receptor ß2 integrin was observed on bovine neutrophils incubated *in vitro* with LKT, LPS and cytokines such as IL-1beta and TNF-alpha, thereby, increasing LKT binding to the leukocytes and subsequent cytotoxicity [102, 129]. The biologic effects of these inflammatory molecules and LKT are morphologically evident in the form of necrotizing bronchopneumonia and formation of neutrophil derived 'oat cells' typical of *M. haemolytica* [48, 88]. To circumvent host defense mechanism of antibody mediated bacterial phagocytosis, *M. haemolytica* has generated a unique method of cleaving IgG delivered to the site of inflammation by using its immunoglobulin proteases [120]. In addition, *in vitro* studies reveal that LKT at sublethal dose is capable of inhibiting concanavalin, pokeweed mitogen, and purified protein derivative induced lymphoid proliferation thereby effecting adaptive immune response [106, 107].

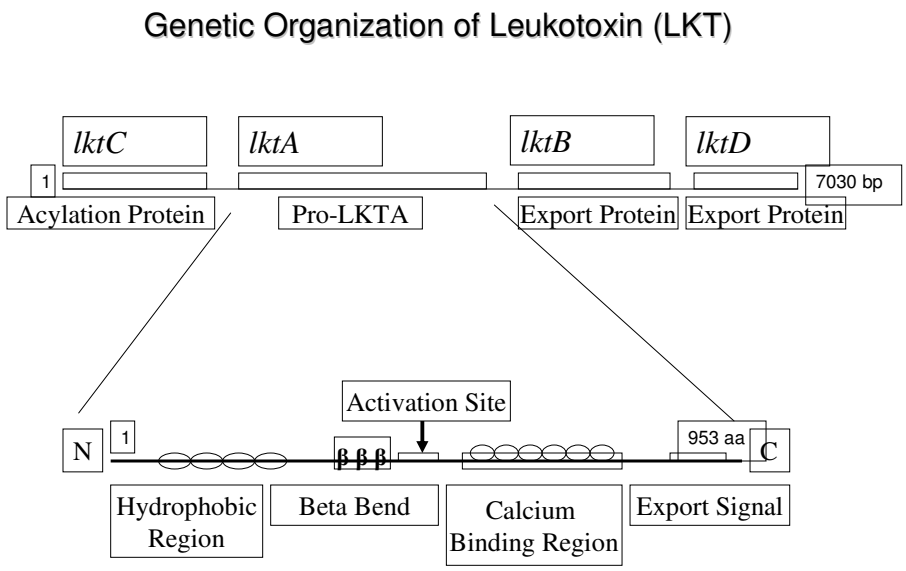

Fig.1. Genetic organization of M. haemolytica S1 leukotoxin (LKT).

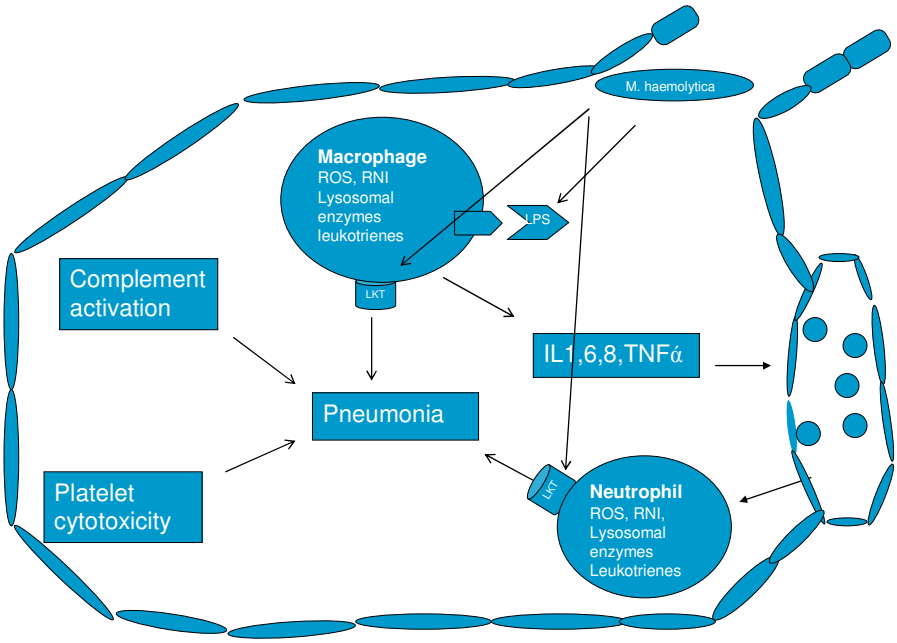

Fig. 2. Oversimplified hypothetical sequence of events followed by inhalation of *M. haemolytica* in the lung. Lipopolysaccharide (LPS) and leukotoxin (LKT) of *M.*

haemolytica origin activate the resident pulmonary macrophages to produce proinflammatory cytokines and undergo oxidative burst. Various cytokines serve as chemoattractant molecules for inflammatory cells. Under the influence of LKT, transmembrane pore are formed in the leukocytes including platelets which result into leakage of cellular constituent including molecules of reactive oxygen and nitrogen species and lysosomal enzymes. These cellular constituents inadvertently damage pulmonary parenchyma and give rise to necrotizing bronchopneumonia typical of *M. haemolytica*. Activation of complement during this process contributes to the damage induced by the bacteria.

References

1. Whiteley LO, Maheswaran SK, Weiss DJ et al. Pasteurella haemolytica A1 and bovine respiratory disease: pathogenesis. J Vet Intern Med 1992;6(1):11-22.

2. Thompson DA, Fraser J, Gilmour NJ. Serotypes of Pasteurella haemolytica in ovine pasteurellosis. Res Vet Sci 1977;22(1):130-1.

3. Gilmour NJ, Thompson DA, Fraser J. The recovery of Pasteurella haemolytica from the tonsils of adult sheep. Res Vet Sci 1974;17(3):413-4.

4. Frank GH. Serotype-specific resistance to nasal colonization by Pasteurella haemolytica in cattle. Am J Vet Res 1985;46(11):2245-8.

5. Frank GH, Smith PC. Prevalence of Pasteurella haemolytica in transported calves. Am J Vet Res 1983;44(6):981-5.

6. Jeyaseelan S, Sreevatsan S, Maheswaran SK. Role of Mannheimia haemolytica leukotoxin in the pathogenesis of bovine pneumonic pasteurellosis. Anim Health Res Rev 2002;3(2):69-82.

7. Grey CL, Thomson RG. Pasteurella haemolytica in the tracheal air of calves. Can J Comp Med 1971;35(2):121-8.

8. Al-Ghamdi GM, Ames TR, Baker JC et al. Serotyping of Mannheimia (Pasteurella) haemolytica isolates from the upper Midwest United States. J Vet Diagn Invest 2000;12(6):576-8.

9. Purdy CW, Raleigh RH, Collins JK et al. Serotyping and enzyme characterization of Pasteurella haemolytica and Pasteurella multocida isolates recovered from pneumonic lungs of stressed feeder calves. Curr Microbiol 1997;34(4):244-9.

10. Ganheim C, Johannisson A, Ohagen P et al. Changes in peripheral blood leucocyte counts and subpopulations after experimental infection with BVDV and/or Mannheimia haemolytica. J Vet Med B Infect Dis Vet Public Health 2005;52(9):380-5.

11. Brown TT,Jr, Ananaba G. Effect of respiratory infections caused by bovine herpesvirus-1 or parainfluenza-3 virus on bovine alveolar macrophage functions. Am J Vet Res 1988;49(9):1447-51.

12. Leite F, Atapattu D, Kuckleburg C et al. Incubation of bovine PMNs with conditioned medium from BHV-1 infected peripheral blood mononuclear cells increases their susceptibility to Mannheimia haemolytica leukotoxin. Vet Immunol Immunopathol 2005;103(3-4):187-93.

13. Kraabel BJ, Miller MW. Effect of simulated stress on susceptibility of bighorn sheep neutrophils to Pasteurella haemolytica leukotoxin. J Wildl Dis 1997;33(3):558-66.

14. Slocombe RF, Malark J, Ingersoll R et al. Importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis in calves. Am J Vet Res 1985;46(11):2253-8.

15. Slocombe RF, Derksen FJ, Robinson NE. Interactions of cold stress and Pasteurella haemolytica in the pathogenesis of pneumonic pasteurellosis in calves: changes in pulmonary function. Am J Vet Res 1984;45(9):1764-70.

16. Slocombe RF, Derksen FJ, Robinson NE et al. Interactions of cold stress and Pasteurella haemolytica in the pathogenesis of pneumonic pasteurellosis in calves: method of induction and hematologic and pathologic changes. Am J Vet Res 1984;45(9):1757-63.

17. Srikumaran S, Kelling CL, Ambagala A. Immune evasion by pathogens of bovine respiratory disease complex. Anim Health Res Rev 2007;8(2):215-29.

18. Rice JA, Carrasco-Medina L, Hodgins DC et al. Mannheimia haemolytica and bovine respiratory disease. Anim Health Res Rev 2007;8(2):117-28.

19. Confer AW, Panciera RJ, Clinkenbeard KD et al. Molecular aspects of virulence of Pasteurella haemolytica. Can J Vet Res 1990;54 Suppl:S48-52.

20. Paulsen DB, Confer AW, Clinkenbeard KD et al. Pasteurella haemolytica lipopolysaccharide-induced arachidonic acid release from and neutrophil adherence to bovine pulmonary artery endothelial cells. Am J Vet Res 1990;51(10):1635-9.

21. Jaramillo L, Diaz F, Hernandez P et al. Purification and characterization of an adhesin from Pasteurella haemolytica. Glycobiology 2000;10(1):31-7.

22. De la Mora A, Trigo F, Jaramillo L et al. The N-acetyl-D-glucosamine specific adhesin from Mannheimia haemolytica activates bovine neutrophils oxidative burst. Vet Immunol Immunopathol 2006;113(1-2):148-56.

23. Lafleur RL, Malazdrewich C, Jeyaseelan S et al. Lipopolysaccharide enhances cytolysis and inflammatory cytokine induction in bovine alveolar macrophages exposed to Pasteurella (Mannheimia) haemolytica leukotoxin. Microb Pathog 2001;30(6):347-57.

24. Lafleur RL, Abrahamsen MS, Maheswaran SK. The biphasic mRNA expression pattern of bovine interleukin-8 in Pasteurella haemolytica lipopolysaccharide-stimulated alveolar macrophages is primarily due to tumor necrosis factor alpha. Infect Immun 1998;66(9):4087-92.

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

26. Mosier DA, Simons KR, Confer AW et al. Pasteurella haemolytica antigens associated with resistance to pneumonic pasteurellosis. Infect Immun 1989;57(3):711-6.

27. Frank GH, Tabatabai LB. Neuraminidase activity of Pasteurella haemolytica isolates. Infect Immun 1981;32(3):1119-22.

28. Otulakowski GL, Shewen PE, Udoh AE et al. Proteolysis of sialoglycoprotein by Pasteurella haemolytica cytotoxic culture supernatant. Infect Immun 1983;42(1):64-70.

29. Highlander SK, Fedorova ND, Dusek DM et al. Inactivation of Pasteurella (Mannheimia) haemolytica leukotoxin causes partial attenuation of virulence in a calf challenge model. Infect Immun 2000;68(7):3916-22.

30. Confer AW, Ayalew S, Panciera RJ et al. Recombinant Mannheimia haemolytica serotype 1 outer membrane protein PlpE enhances commercial M. haemolytica vaccine-induced resistance against serotype 6 challenge. Vaccine 2006;24(13):2248-55.

31. Straus DC, Unbehagen PJ, Purdy CW. Neuraminidase production by a Pasteurella haemolytica A1 strain associated with bovine pneumonia. Infect Immun 1993;61(1):253-9.

32. Clarke JM, Morton RJ, Clarke CR et al. Development of an ex vivo model to study adherence of Mannheimia haemolytica serovar 1 to mucosal tissues of the respiratory tract of cattle. Am J Vet Res 2001;62(5):805-11.

33. Highlander SK. Molecular genetic analysis of virulence in Mannheimia (pasteurella) haemolytica. Front Biosci 2001;6:D1128-50.

34. Briggs RE, Frank GH. Increased elastase activity in nasal mucus associated with nasal colonization by Pasteurella haemolytica in infectious bovine rhinotracheitis virus-infected calves. Am J Vet Res 1992;53(5):631-5.

35. Retzer MD, Yu R, Zhang Y et al. Discrimination between apo and iron-loaded forms of transferrin by transferrin binding protein B and its N-terminal subfragment. Microb Pathog 1998;25(4):175-80.

36. Gioia J, Qin X, Jiang H et al. The genome sequence of Mannheimia haemolytica A1: insights into virulence, natural competence, and Pasteurellaceae phylogeny. J Bacteriol 2006;188(20):7257-66.

37. Malazdrewich C, Ames TR, Abrahamsen MS et al. Pulmonary expression of tumor necrosis factor alpha, interleukin-1 beta, and interleukin-8 in the acute phase of bovine pneumonic pasteurellosis. Vet Pathol 2001;38(3):297-310.

38. Davies RL, Arkinsaw S, Selander RK. Evolutionary genetics of Pasteurella haemolytica isolates recovered from cattle and sheep. Infect Immun 1997;65(9):3585-93.

39. Davies RL, Donachie W. Intra-specific diversity and host specificity within Pasteurella haemolytica based on variation of capsular polysaccharide, lipopolysaccharide and outer-membrane proteins. Microbiology 1996;142 (Pt 7)(Pt 7):1895-907.

40. Davies RL, Paster BJ, Dewhirst FE. Phylogenetic relationships and diversity within the Pasteurella haemolytica complex based on 16S rRNA sequence comparison and outer membrane protein and lipopolysaccharide analysis. Int J Syst Bacteriol 1996;46(3):736-44.

41. Lacroix RP, Duncan JR, Jenkins RP et al. Structural and serological specificities of Pasteurella haemolytica lipopolysaccharides. Infect Immun 1993;61(1):170-81.

42. Ali Q, Davies RL, Parton R et al. Lipopolysaccharide heterogeneity in Pasteurella haemolytica isolates from cattle and sheep. J Gen Microbiol 1992;138(10):2185-95.

43. Li J, Clinkenbeard KD. Lipopolysaccharide complexes with Pasteurella haemolytica leukotoxin. Infect Immun 1999;67(6):2920-7.

44. Whiteley LO, Maheswaran SK, Weiss DJ et al. Immunohistochemical localization of Pasteurella haemolytica A1-derived endotoxin, leukotoxin, and capsular polysaccharide in experimental bovine Pasteurella pneumonia. Vet Pathol 1990;27(3):150-61.

45. Yoo HS, Maheswaran SK, Lin G et al. Induction of inflammatory cytokines in bovine alveolar macrophages following stimulation with Pasteurella haemolytica lipopolysaccharide. Infect Immun 1995;63(2):381-8.

46. Yoo HS, Rutherford MS, Maheswaran SK et al. Induction of nitric oxide production by bovine alveolar macrophages in response to Pasteurella haemolytica A1. Microb Pathog 1996;20(6):361-75.

47. Caswell JL, Middleton DM, Sorden SD et al. Expression of the neutrophil chemoattractant interleukin-8 in the lesions of bovine pneumonic pasteurellosis. Vet Pathol 1998;35(2):124-31.

48. Maheswaran SK, Weiss DJ, Kannan MS et al. Effects of Pasteurella haemolytica A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals. Vet Immunol Immunopathol 1992;33(1-2):51-68.

49. Wessely-Szponder J, Urban-Chmiel R, Wernicki A et al. Effect of leukotoxin of Mannheimia haemolytica and LPS of E. coli on secretory response of bovine neutrophils in vitro. Pol J Vet Sci 2005;8(2):99-105.

50. Radi ZA, Caverly JM, Dixon RA et al. Effects of the synthetic selectin inhibitor TBC1269 on tissue damage during acute Mannheimia haemolytica-induced pneumonia in neonatal calves. Am J Vet Res 2001;62(1):17-22.

51. Hsuan SL, Kannan MS, Jeyaseelan S et al. Pasteurella haemolytica leukotoxin and endotoxin induced cytokine gene expression in bovine alveolar macrophages requires NF-kappaB activation and calcium elevation. Microb Pathog 1999;26(5):263-73.

52. Paulsen DB, Confer AW, Clinkenbeard KD et al. Pasteurella haemolytica lipopolysaccharide-induced cytotoxicity in bovine pulmonary artery endothelial monolayers: inhibition by indomethacin. Vet Pathol 1995;32(2):173-83.

53. Morsey MA, Van-Kessel AG, Mori Y et al. Cytokine profiles following interaction between bovine alveolar macrophages and Pasteurella haemolytica. Microb Pathog 1999;26(6):325-31.

54. Brogden KA, Cutlip RC, Lehmkuhl HD. Complexing of bacterial lipopolysaccharide with lung surfactant. Infect Immun 1986;52(3):644-9.

55. Confer AW, Simons KR. Effects of Pasteurella haemolytica lipopolysaccharide on selected functions of bovine leukocytes. Am J Vet Res 1986;47(1):154-7.

56. Emau P, Giri SN, Bruss ML. Effects of smooth and rough Pasteurella haemolytica lipopolysaccharides on plasma cyclic-nucleotides and free fatty acids in calves. Vet Microbiol 1987;15(4):279-92.

57. Confer AW, Panciera RJ, Mosier DA. Serum antibodies to Pasteurella haemolytica lipopolysaccharide: relationship to experimental bovine pneumonic pasteurellosis. Am J Vet Res 1986;47(5):1134-8.

58. Corstvet RE, Gentry MJ, Newman PR et al. Demonstration of age-dependent capsular material on Pasteurella haemolytica serotype 1. J Clin Microbiol 1982;16(6):1123-6.

59. Adlam C, Knights JM, Mugridge A et al. Purification, characterization and immunological properties of the capsular polysaccharide of Pasteurella haemolytica serotype T15: its identity with the K62 (K2ab) capsular polysaccharide of Escherichia coli and the capsular polysaccharide of Neisseria meningitidis serogroup H. J Gen Microbiol 1985;131(8):1963-72.

60. Adlam C, Knights JM, Mugridge A et al. Purification, characterization and immunological properties of the serotype-specific capsular polysaccharide of

Pasteurella haemolytica (serotype A1) organisms. J Gen Microbiol 1984;130(9):2415-26.

61. Morck DW, Watts TC, Acres SD et al. Electron microscopic examination of cells of Pasteurella haemolytica-A1 in experimentally infected cattle. Can J Vet Res 1988;52(3):343-8.

62. Czuprynski CJ, Noel EJ, Adlam C. Interaction of bovine alveolar macrophages with Pasteurella haemolytica A1 in vitro: modulation by purified capsular polysaccharide. Vet Microbiol 1991;26(4):349-58.

63. Czuprynski CJ, Noel EJ, Adlam C. Modulation of bovine neutrophil antibacterial activities by Pasteurella haemolytica A1 purified capsular polysaccharide. Microb Pathog 1989;6(2):133-41.

64. Chae CH, Gentry MJ, Confer AW et al. Resistance to host immune defense mechanisms afforded by capsular material of Pasteurella haemolytica, serotype 1. Vet Microbiol 1990;25(2-3):241-51.

65. Wilson CF, Sutherland AD, Inglis L et al. Characterisation and biological activity of monoclonal antibodies specific for Pasteurella haemolytica A1 capsule and lipopolysaccharide. Vet Microbiol 1992;31(2-3):161-8.

66. Pandher K, Murphy GL, Confer AW. Identification of immunogenic, surface-exposed outer membrane proteins of Pasteurella haemolytica serotype 1. Vet Microbiol 1999;65(3):215-26.

67. Ogunnariwo JA, Woo TK, Lo RY et al. Characterization of the Pasteurella haemolytica transferrin receptor genes and the recombinant receptor proteins. Microb Pathog 1997;23(5):273-84.

68. Kirby SD, Lainson FA, Donachie W et al. The Pasteurella haemolytica 35 kDa iron-regulated protein is an FbpA homologue. Microbiology 1998;144 (Pt 12)(Pt 12):3425-36.

69. Iovane G, Galdiero M, Vitiello M et al. Effect of Pasteurella haemolytica outer membrane proteins on bovine neutrophils. FEMS Immunol Med Microbiol 1998;20(1):29-36.

70. Shewen PE, Wilkie BN. Vaccination of calves with leukotoxic culture supernatant from Pasteurella haemolytica. Can J Vet Res 1988;52(1):30-6.

71. Pandher K, Confer AW, Murphy GL. Genetic and immunologic analyses of PlpE, a lipoprotein important in complement-mediated killing of Pasteurella haemolytica serotype 1. Infect Immun 1998;66(12):5613-9.

72. Davies RL, Lee I. Sequence diversity and molecular evolution of the heat-modifiable outer membrane protein gene (ompA) of Mannheimia(Pasteurella) haemolytica, Mannheimia glucosida, and Pasteurella trehalosi. J Bacteriol 2004;186(17):5741-52.

73. Marcatili A, D'Isanto M, Galdiero M et al. Role of Pasteurella multocida, Pasteurella haemolytica and Salmonella typhimurium porins on inducible nitric oxide release by murine macrophages. Res Microbiol 2000;151(3):217-28.

74. Jeyaseelan S, Kannan MS, Briggs RE et al. Mannheimia haemolytica leukotoxin activates a nonreceptor tyrosine kinase signaling cascade in bovine leukocytes, which induces biological effects. Infect Immun 2001;69(10):6131-9.

75. Cruz WT, Young R, Chang YF et al. Deletion analysis resolves cell-binding and lytic domains of the Pasteurella leukotoxin. Mol Microbiol 1990;4(11):1933-9.

76. Yin Y, Zhang F, Ling V et al. Structural analysis and comparison of the C-terminal transport signal domains of hemolysin A and leukotoxin A. FEBS Lett 1995;366(1):1-5.

77. Crosby JA, Kachlany SC. TdeA, a TolC-like protein required for toxin and drug export in Aggregatibacter (Actinobacillus) actinomycetemcomitans. Gene 2007;388(1-2):83-92.

78. Welch RA, Forestier C, Lobo A et al. The synthesis and function of the Escherichia coli hemolysin and related RTX exotoxins. FEMS Microbiol Immunol 1992;5(1-3):29-36.

79. Shewen PE, Wilkie BN. Evidence for the Pasteurella haemolytica cytotoxin as a product of actively growing bacteria. Am J Vet Res 1985;46(5):1212-4.

80. Highlander SK, Chidambaram M, Engler MJ et al. DNA sequence of the Pasteurella haemolytica leukotoxin gene cluster. DNA 1989;8(1):15-28.

81. Thumbikat P, Briggs RE, Kannan MS et al. Biological effects of two genetically defined leukotoxin mutants of Mannheimia haemolytica. Microb Pathog 2003;34(5):217-26.

82. Sun Y, Clinkenbeard KD, Cudd LA et al. Correlation of Pasteurella haemolytica leukotoxin binding with susceptibility to intoxication of lymphoid cells from various species. Infect Immun 1999;67(12):6264-9.

83. Forestier C, Welch RA. Identification of RTX toxin target cell specificity domains by use of hybrid genes. Infect Immun 1991;59(11):4212-20.

84. Narayanan SK, Nagaraja TG, Chengappa MM et al. Leukotoxins of gram-negative bacteria. Vet Microbiol 2002;84(4):337-56.

85. Clinkenbeard KD, Mosier DA, Confer AW. Effects of Pasteurella haemolytica leukotoxin on isolated bovine neutrophils. Toxicon 1989;27(7):797-804.

86. Thumbikat P, Dileepan T, Kannan MS et al. Mechanisms underlying Mannheimia haemolytica leukotoxin-induced oncosis and apoptosis of bovine alveolar macrophages. Microb Pathog 2005;38(4):161-72.

87. Atapattu DN, Czuprynski CJ. Mannheimia haemolytica leukotoxin induces apoptosis of bovine lymphoblastoid cells (BL-3) via a caspase-9-dependent mitochondrial pathway. Infect Immun 2005;73(9):5504-13.

88. Maheswaran SK, Kannan MS, Weiss DJ et al. Enhancement of neutrophil-mediated injury to bovine pulmonary endothelial cells by Pasteurella haemolytica leukotoxin. Infect Immun 1993;61(6):2618-25.

89. Tatum FM, Briggs RE, Sreevatsan SS et al. Construction of an isogenic leukotoxin deletion mutant of Pasteurella haemolytica serotype 1: characterization and virulence. Microb Pathog 1998;24(1):37-46.

90. Saadati M, Gibbs HA, Parton R et al. Characterisation of the leukotoxin produced by different strains of Pasteurella haemolytica. J Med Microbiol 1997;46(4):276-84.

91. Davies RL, Baillie S. Cytotoxic activity of Mannheimia haemolytica strains in relation to diversity of the leukotoxin structural gene lktA. Vet Microbiol 2003;92(3):263-79.

92. Dassanayake RP, Maheswaran SK, Srikumaran S. Monomeric expression of bovine beta2-integrin subunits reveals their role in Mannheimia haemolytica leukotoxin-induced biological effects. Infect Immun 2007;75(10):5004-10.

93. Dassanayake RP, Shanthalingam S, Davis WC et al. Mannheimia haemolytica leukotoxin-induced cytolysis of ovine (Ovis aries) leukocytes is mediated by CD18, the beta subunit of beta2-integrins. Microb Pathog 2007;42(5-6):167-73.

94. Lawrence PK, Nelson WR, Liu W et al. beta(2) integrin Mac-1 is a receptor for Mannheimia haemolytica leukotoxin on bovine and ovine leukocytes. Vet Immunol Immunopathol 2008.

95. Albelda SM, Buck CA. Integrins and other cell adhesion molecules. FASEB J 1990;4(11):2868-80.

96. Jeyaseelan S, Hsuan SL, Kannan MS et al. Lymphocyte function-associated antigen 1 is a receptor for Pasteurella haemolytica leukotoxin in bovine leukocytes. Infect Immun 2000;68(1):72-9.

97. Liu W, Brayton KA, Davis WC et al. Mannheimia (Pasteurella) haemolytica leukotoxin utilizes CD18 as its receptor on bighorn sheep leukocytes. J Wildl Dis 2007;43(1):75-81.

98. Dileepan T, Thumbikat P, Walcheck B et al. Recombinant expression of bovine LFA-1 and characterization of its role as a receptor for Mannheimia haemolytica leukotoxin. Microb Pathog 2005;38(5-6):249-57.

99. Gopinath RS, Ambagala TC, Deshpande MS et al. Mannheimia (Pasteurella) haemolytica leukotoxin binding domain lies within amino acids 1 to 291 of bovine CD18. Infect Immun 2005;73(9):6179-82.

100. Lawrence PK, Dassanayake RP, Knowles DP et al. Transfection of non-susceptible cells with Ovis aries recombinant lymphocyte function-associated antigen 1 renders susceptibility to Mannheimia haemolytica leukotoxin. Vet Microbiol 2007;125(1-2):91-9.

101. Czuprynski CJ, Hamilton HL, Noel EJ. Ingestion and killing of Pasteurella haemolytica A1 by bovine neutrophils in vitro. Vet Microbiol 1987;14(1):61-74.

102. Leite F, Gyles S, Atapattu D et al. Prior exposure to Mannheimia haemolytica leukotoxin or LPS enhances beta(2)-integrin expression by bovine neutrophils and augments LKT cytotoxicity. Microb Pathog 2003;34(6):267-75.

103. Leite F, Kuckleburg C, Atapattu D et al. BHV-1 infection and inflammatory cytokines amplify the interaction of Mannheimia haemolytica leukotoxin with bovine peripheral blood mononuclear cells in vitro. Vet Immunol Immunopathol 2004;99(3-4):193-202.

104. Dileepan T, Thumbikat P, Walcheck B et al. Recombinant expression of bovine LFA-1 and characterization of its role as a receptor for Mannheimia haemolytica leukotoxin. Microb Pathog 2005;38(5-6):249-57.

105. Dileepan T, Kannan MS, Walcheck B et al. Integrin-EGF-3 domain of bovine CD18 is critical for Mannheimia haemolytica leukotoxin species-specific susceptibility. FEMS Microbiol Lett 2007;274(1):67-72.

106. Majury AL, Shewen PE. The effect of Pasteurella haemolytica A1 leukotoxic culture supernate on the in vitro proliferative response of bovine lymphocytes. Vet Immunol Immunopathol 1991;29(1-2):41-56.

107. Majury AL, Shewen PE. Preliminary investigation of the mechanism of inhibition of bovine lymphocyte proliferation by Pasteurella haemolytica A1 leukotoxin. Vet Immunol Immunopathol 1991;29(1-2):57-68.

108. Clinkenbeard KD, Mosier DA, Confer AW. Transmembrane pore size and role of cell swelling in cytotoxicity caused by Pasteurella haemolytica leukotoxin. Infect Immun 1989;57(2):420-5.

109. Clinkenbeard KD, Mosier DA, Confer AW. Effects of Pasteurella haemolytica leukotoxin on isolated bovine neutrophils. Toxicon 1989;27(7):797-804.

110. Menestrina G, Mackman N, Holland IB et al. Escherichia coli haemolysin forms voltage-dependent ion channels in lipid membranes. Biochim Biophys Acta 1987;905(1):109-17.

111. Hsuan SL, Kannan MS, Jeyaseelan S et al. Pasteurella haemolytica A1-derived leukotoxin and endotoxin induce intracellular calcium elevation in bovine alveolar macrophages by different signaling pathways. Infect Immun 1998;66(6):2836-44.

112. Cheryk LA, Hooper-McGrevy KE, Gentry PA. Alterations in bovine platelet function and acute phase proteins induced by Pasteurella haemolytica A1. Can J Vet Res 1998;62(1):1-8.

113. Nyarko KA, Coomber BL, Mellors A et al. Bovine platelet adhesion is enhanced by leukotoxin and sialoglycoprotease isolated from Pasteurella haemolytica A1 cultures. Vet Microbiol 1998;61(1-2):81-91.

114. Clinkenbeard KD, Upton ML. Lysis of bovine platelets by Pasteurella haemolytica leukotoxin. Am J Vet Res 1991;52(3):453-7.

115. Czuprynski CJ, Noel EJ, Ortiz-Carranza O et al. Activation of bovine neutrophils by partially purified Pasteurella haemolytica leukotoxin. Infect Immun 1991;59(9):3126-33.

116. Adusu TE, Conlon PD, Shewen PE et al. Pasteurella haemolytica leukotoxin induces histamine release from bovine pulmonary mast cells. Can J Vet Res 1994;58(1):1-5.

117. Yoo HS, Rajagopal BS, Maheswaran SK et al. Purified Pasteurella haemolytica leukotoxin induces expression of inflammatory cytokines from bovine alveolar macrophages. Microb Pathog 1995;18(4):237-52.

118. Wilkie BN, Markham RJ. Bronchoalveolar washing cells and immunoglobulins of clinically normal calves. Am J Vet Res 1981;42(2):241-3.

119. Pringle JK, Viel L, Shewen PE et al. Bronchoalveolar lavage of cranial and caudal lung regions in selected normal calves: cellular, microbiological, immunoglobulin, serological and histological variables. Can J Vet Res 1988;52(2):239-48.

120. Lee CW, Shewen PE. Evidence of bovine immunoglobulin G1 (IgG1) protease activity in partially purified culture supernate of Pasteurella haemolytica A1. Can J Vet Res 1996;60(2):127-32.

121. Shewen PE, Lee CW, Perets A et al. Efficacy of recombinant sialoglycoprotease in protection of cattle against pneumonic challenge with Mannheimia (Pasteurella) haemolytica A1. Vaccine 2003;21(17-18):1901-6.

122. Abdullah KM, Lo RY, Mellors A. Cloning, nucleotide sequence, and expression of the Pasteurella haemolytica A1 glycoprotease gene. J Bacteriol 1991;173(18):5597-603.

123. Frank GH, Tabatabai LB. Neuraminidase activity of Pasteurella haemolytica isolates. Infect Immun 1981;32(3):1119-22.

124. Krivan HC, Roberts DD, Ginsburg V. Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids. Proc Natl Acad Sci U S A 1988;85(16):6157-61.

125. Gottschalka A. Correlation between composition, structure, shape and function of a salivary mucoprotein. Nature 1960;186:949-51.

126. Soong G, Muir A, Gomez MI et al. Bacterial neuraminidase facilitates mucosal infection by participating in biofilm production. J Clin Invest 2006;116(8):2297-305.

127. Clinkenbeard KD, Mosier DA, Timko AL et al. Effects of Pasteurella haemolytica leukotoxin on cultured bovine lymphoma cells. Am J Vet Res 1989;50(2):271-5.

128. Clarke CR, Confer AW, Mosier DA. In vivo effect of Pasteurella haemolytica infection on bovine neutrophil morphology. Am J Vet Res 1998;59(5):588-92.

129. Stevens P, Czuprynski C. Dissociation of cytolysis and monokine release by bovine mononuclear phagocytes incubated with Pasteurella haemolytica partially purified leukotoxin and lipopolysaccharide. Can J Vet Res 1995;59(2):110-7

CHAPTER III

Cytokine mRNA modulation in bovine alveolar macrophages challenged with the wild type and leukotoxin mutant *Mannheimia haemolytica*

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Leukotoxin (LKT) and lipopolysaccharide (LPS) are two important virulence factors associated with *Mannheimia haemolytica*. The importance of these virulence factors is well-documented by multiple *in vitro* studies using purified LPS and/or LKT. Studies using whole bacteria containing all virulence factors would provide better insight into the pathogenesis of shipping fever. Unfortunately, these studies are limited. Bovine alveolar macrophages (BAM) were challenged with wild type (wt) and LKT-deficient (*lkt*$^-$) *M. haemolytica* at the concentration of 1 bacterium per BAM. The effects on macrophage function were measured by quantifying the cytokine response using ELISA and real-time RT-PCR. Increased protein secretion of TNFα and IL-10 was observed in the supernatant obtained from BAM-challenged with *lkt*$^-$ strain compared to wt-challenged BAM. Similarly, using real-time RT-PCR, increased mRNA expression of cytokines TNFα, IL-1ß, IL-8, IL-10 and IL-12 was observed in *lkt*$^-$ strain-challenged BAM compared to the wt-challenged BAM. An overall significant effect for all cytokines combined together was observed between the two groups, but significant differences were not observed between the two groups for individual cytokines. BAM challenged with wt strain exhibited, in average, 43% more cytotoxicity, quantified using MTT assay, than *lkt*$^-$ -challenged BAM ($p < 0.01$) indicating cell-cytotoxicity plays a role in decreased cytokine responses in wt-challenged BAM compared to *lkt*$^-$ -challenged BAM *in vitro*. These results suggest that multiple cytokines are involved and that virulence factors other than LKT play an important role in the pathogenesis of shipping fever.

Key words: Bovine alveolar macrophages, cytokine, leukotoxin, *Mannheimia haemolytica*, real-time PCR, Shipping fever

Introduction

Mannheimia haemolytica is the major cause of fibrinous and necrotizing bronchopneumonia of cattle termed bovine pneumonic pasteurellosis (BPP) or shipping fever. BPP has a significant economic impact costing more than $ 1 billion annually to the US cattle industry alone. [1] Despite improved management practices and extensive use of vaccination programs, BPP continues to be a major cause of losses in feedlot cattle [2]. Several virulence factors of *M. haemolytica* promote host-pathogen interactions by helping the bacterial colonization of cattle lungs and subsequently the development of pneumonia. Leukotoxin (LKT) and lipopolysaccharide (LPS) are two critically important virulence factors and their roles in the pathogenesis of shipping fever are well documented [3]. Other virulence factors with less documented pathogenic roles include capsule, fimbriae, iron-regulated proteins, outer membrane proteins, neuraminidase, and a neutral glycoprotease [3, 4].

Since its initial discovery, LKT has been subject of intense investigation relative to its role in the pathogenesis of shipping fever. LKT follows a ruminant species-specific, dose-dependent activation-inhibition paradox on bovine leucocytes [5, 6]. At low concentrations, LKT activates neutrophils and macrophages resulting in respiratory burst, degranulation, cytokine release from macrophages, reduced lymphoblastogenesis, and histamine release from mast cells[5, 7]. At high concentration, LKT stimulates cells to undergo apoptosis whereas at highest concentration, LKT causes the formation of transmembrane pores and subsequent cell necrosis [8-10].

Most of the knowledge that has been generated about the role of LKT and LPS in the pathogenesis of shipping fever comes from limited *in vivo* and *in vitro* studies using purified or partially purified LKT alone or in combination with LPS [11, 12]. Because LKT and LPS functionally interact and exhibit synergism, their roles are better understood when used in conjunction with each other[13]. Results obtained from *in vivo* studies indicate that inoculation of calves with a LKT-deficient mutant *M. haemolytica* cause a decreased mortality and lung lesions compared to the calves challenged with the wild-type strain [14, 15]. Multiple cytokines are involved in the pathogenesis of shipping

fever and it is possible that altered pulmonary cytokine responses are responsible for the reduced virulence of LKT-deficient mutant *M. haemolytica.* A comprehensive understanding of cytokines expression would enable better assessment of the roles cytokines play. Multiple studies support the role of cytokines in the pathogenesis of BPP[16]. We identified cytokines TNFα, IL-1ß, IL-6 and IL-8 because these are pleiotropic proinflammatory molecules produced by a variety of cells and play significant role in innate immunity[16, 17]. The cytokines IL-10 and IL-12 were selected for measurement because they are involved principally in regulating adaptive immune response.

We investigated the role of LKT in modulating cytokine gene and protein expression in bovine alveolar macrophages (BAM) by using isogenic LKT-deficient (*lkt*$^-$) and wild type (wt) *M. haemolytica* strains. Our hypothesis was that the modulation of cytokine mRNA response observed in the acute and adaptive phase in BAM is modulated by LKT. The wt strain was isolated from a pneumonic bovine lung, and isogenic *lkt*$^-$ strain was previously prepared by the allelic replacement of the entire *lktA* and partial *lktC* gene with beta-lactamase enzyme gene[18]. Western blot data supported that no LKT was produced by *lkt*$^-$ *M. haemolytica* [18]. Additionally, in contrast to the wt strain, no zone of peripheral hemolysis was associated with the colonies of *lkt*$^-$ strain on sheep blood agar [18]. The objective of this study was to evaluate the effect of LKT on cytokine mRNA expression in BAM following *in vitro* challenge with wt and *lkt*$^-$ strain *M. haemolytica*. We used BAM because they represent the front line of host defense against inhaled microorganisms that reach the lung, regulate the early inflammatory response including infiltration of neutrophils, and serve as a link between innate and adaptive immunity [19].

Material and methods

a. Alveolar macrophage collection and preparation

Multiple lungs were obtained from freshly euthanized animals from the slaughter house. All lung lobes were lavaged with 1 L total normal saline containing 4% fetal bovine serum (FBS) and 1% penicillin-streptomycin and amphotericin B (Cambrex, Walkersville, MD, USA) using an endotracheal tube. Retrieval volume average 500-600 mL per lung. The lavage fluid was immediately delivered to the laboratory on ice for BAM isolation. The recovered bronchiolar alveolar lavage (BAL) fluid was pelleted and washed twice at 500 x g for 7 minutes with 20 ml of phosphate buffer saline (PBS). The washed pellet was resuspended in 3 ml RPMI-1640 media (Hyclone, Hyq RPMI-1640) enriched with 10% FBS, 1% L glutamine, 1% HEPES, 1% sodium bicarbonate, and 10,000 IU/ml penicillin-streptomycin media and viable cells counted using a hemocytometer by Trypan blue exclusion method. Subsequently, 2 ml of 2.5 x 10^6 BAM/ml/well were incubated at 37ºC in a 6 well plate for 3 hours. Non-adherent cells were removed after 3 hours by changing the medium and gently washing the cells. The cells were further incubated for 36-40 hours prior to challenge. Medium was changed once every 24 hours without disturbing the cells. The adherent cell population of BAL consisted of approximately 95%-100% BAM as observed based on their morphology. Following 36-40 hour incubation, BAM were challenged with 2 ml of enriched RPMI, without penicillin-streptomycin, containing 2.5 x 10^6 wt and *lkt*$^-$ -strain/ml. The supernatant was harvested at 0, 1,2,4,6,8,12, and 24 hours post incubation and stored at -20 ◦C for further use.

b. Bacterial inoculum

Both wt and *lkt*$^-$ *M. haemolytica* cultures had nearly identical growth curves and were prepared similarly. Briefly, bacteria were grown overnight on brain heart infusion (BHI) agar containing 5% sheep blood (Hardy Diagnostics, Santa Maria, CA) with and without ampicillin (10ug/ml) for *lkt*$^{--}$ and wt cultures, respectively. Multiple colonies were subsequently transferred to 150 ml of BHI broth and incubated for 6 hours at 37ºC at 70 oscillations per minute (opm). The broth culture was centrifuged twice at 8000 x g for 15 minutes. The bacterial pellet was washed with 10 ml PBS. After a second centrifugation,

the pellet was resuspended in RPMI media or PBS and the concentration was quantified using spectrophotometer (Pharmacia, Ultraspec 2000, Haverhill, MA). In our previous experiments, the OD of 0.65-0.72 at 650 nm corresponded to 10^9 cfu/ml[20]. The exact concentration of the viable bacterial suspension was subsequently confirmed by standard colony count at various dilutions on BHI blood agar.

c. Leukotoxin production and partial purification: Both wt and *lkt*$^-$ strains were grown on BHI supplemented with 5% sheep blood agar. A single colony was suspended in BHI broth and incubated overnight at 37ºC in a shaking incubator at 160 opm. The culture was subsequently resuspended in 1L RPMI 1640 (R7509 Sigma) and incubated at 37ºC at 160 opm. The culture was centrifuged at 3800 x g for 20 minutes. The supernatant obtained was filtered using 0.2 µm filters (Fisher 167-0020). The concentrate was repeatedly centrifuged (Millipore, Amicon ultra-15) at 2000 x g for 15 minutes. The culture supernatant containing semi purified LKT was aliquoted and stored at -86°C for further use in western blot.

d. Western blot: To verify the authenticity of the *lkt*$^-$ strain, a western blot was performed as described previously [18]. Briefly, 500 µl of final buffer mix (containing 10% glycerol, 2.5 % SDS, and 0.1% bromophenol blue) was added to an equal volume of semi purified LKT concentrate and boiled for 5 minutes. Twenty µl of heated LKT concentrate was loaded on a 10% polyacrylamide gel, electrophoresed and transferred to nitrocellulose membranes. The membrane was blocked, washed and probed with a non-neutralizing anti-LKT monoclonal antibody (MM 605 kindly provided by Dr. S. Srikumaran, Washington state University, Pullman, Washington) at 37ºC for 1 hour. The blot was finally washed and probed with alkaline phosphatase conjugated secondary anti-murine antibody at 37ºC for 1 hour and visualized using BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium).

e. Bacterial concentration: Bovine alveolar macrophages challenged with *M. haemolytica* are activated at low dose whereas cytotoxicity is observed at higher dose;

therefore, a pilot experiment was conducted to determine the optimum concentration of bacteria required to challenge BAM [5]. Different concentrations of bacteria per cell (0.1 bacteria/cell, 1 bacterium/cell, 10 bacteria/cell, and 100 bacteria/cell) stimulated BAM to produce similar concentration of TNFα protein in the cell culture medium (data not shown). Based on these results, concentration of 1 bacterium per macrophage was used to challenge BAM.

f. Real-time RT-PCR: mRNA expression of multiple cytokines was determined by real time PCR. Briefly, the total RNA was extracted using the guanidine thiocyanate technique as described previously[21] and cDNA was generated by utilizing reverse transcriptase kit (QuantiTect Rev. Transcription Kit, Qiagen). Briefly, RNAstat-60 (Tel-Test B, Friendswood, TX) was added to the pelleted cells and the cells lysed by pipetting up and down multiple times. The RNA was subsequently isolated according to the kits instructions, measured with spectrophotometer, and adjusted to 0.1 µg/µl with Diethyl pyrocarbonate (DEPC) treated water. Real-time RT-PCRs were performed in individual 500 µl Eppendorf tubes containing 12.5 µl of 1x TaqMan Universal PCR master mix, 1.5 µM each primer, 10 µl water, and 2·0 µl sample cDNA in a final volume of 26 µl. PCR amplification and detection were performed on an ABI Prism 7000 Sequence Detection System using the following cycling conditions: 50 °C for 2 min, 95 °C for 10 min and 40 cycles of 94 °C for 15 sec, 55°C for 30 sec, and 72 °C for 30 sec. All RT-PCRs were carried out in duplicate with appropriate controls run on each plate. Bovine beta actin mRNA was used as endogenous reference mRNA. The sequences of primers used in this study were previously published[22]. The geometric mean was calculated as [(ct value of a cytokine from a treated group – ct value of actin from that treated group) – (ct value that cytokine from untreated group – ct value of actin from untreated group).

g. Cytotoxicity assay: The susceptibility of the BAM to *M. haemolytica* LKT-mediated cytolysis was determined by a previously described cytotoxicity assay using Thiazolyl Blue Tetrazolium Bromide (MTT, Sigma, St Louis, MO, USA). Briefly, 100 µl of 2.5 x 10^6 BAM/ml were incubated with the same number of wt and *lkt*$^-$ strain in a 96-well flat bottom microtiter plate. Following incubation, the culture plate was centrifuged at 2000 rpm for 10 minutes. The supernatant was removed and cells were incubated with 200 µl

of RPMI media enriched with antibiotics for 1 hour. The microtiter plate was centrifuged at 2000 rpm for 10 minutes. Following removal of supernatant, 100 µl of RPMI and 20 µl of MTT solutions were added to each well. The plate was then incubated on slow shaker at 37ºC for 2-3 hours. Following incubation plate was centrifuged again at 6000 xg for 10 minutes and the supernatant was removed by pipetting. The MTT crystals were dissolved in 150 µl of isopropanol by repeated gentle pipetting. Absorbance was determined using microplate reader at 450 nm. The percent cytotoxicity was calculated as [1-(optical density of cells incubated with *M. haemolytica*/optical density of cells not incubated with *M. haemolytica*)] x 100.

h. ELISAs: A TNFα ELISA kit (Endogen, Bovine TNF-alpha screening set, Rockford, IL, USA) was used according to the manufacturer's instructions. ELISA for IL-10 was performed as previously described [25]. ELISA kits for other bovine cytokines are not available.

g. Data analysis: Mixed linear models were used to perform the hypothesis testing. Hurvich and Tsai's Criterion (AICC) [23] was used to select the best model to fit the observed data. Kenward-Roger method was applied to adjust for small sample size[24]. Logarithm transformation was applied on cytokine mRNA expression and the present standard errors for mRNA expression were in logarithm transformed scales.

Results

1. LKT-deficient *M. haemolytica* did not produce LKT

The monoclonal antibody (MM 605) recognized the 102 kDa LKT band and associated fragmented bands in the supernatant obtained from the culture of wt *M. haemolytica,* whereas a similar band was not detected in the culture supernatant obtained from *lkt*$^-$ *M. haemolytica* (Fig. 1). Moreover, the growth of the wt strain on 5% sheep blood agar was accompanied by a definite zone of hemolysis surrounding the colonies, whereas no zone of hemolysis was associated with the growth of *lkt*$^-$ strain (data not shown).

2. LKT-deficient *M. haemolytica* induced increased cytokine protein secretion from bovine alveolar macrophages

Bovine alveolar macrophages challenged with *lkt*$^-$ -strain produced increased quantities of TNFα ($p < 0.001$) and IL-10 ($p = 0.002$) protein in the culture supernatant compared to the wt strain. The IL-10 protein was detected in the supernatant obtained from *lkt*$^-$ -challenged BAM starting at 1 hour (mean = 0.034 pg/ml; standard error (SE) = 0.017) post-challenge, it increased until 8 hours (mean = 0.114 pg/ml; SE = 0.018), and then peaked at 12 hours (mean = 0.300 pg/ml; SE = 0.231), whereas no significant ($p = 0.500$) amount of IL-10 protein was detected in the culture supernatant obtained from wt-challenged BAM (Fig. 2). Significant amount of TNFα protein was demonstrated in the culture supernatant soon after challenge of BAM with both wt and *lkt*$^-$ strains ($p = 0.024$ and < 0.001, respectively). TNFα protein peaked at 4 hours (mean = 411 pg/ml; SE = 99) in *lkt*$^-$ -challenged BAM (Fig. 3). In wt-challenged BAM TNFα protein peaked at 8 hours (mean = 178 pg/ml; SE = 73) and returned to base level by 24 hours (Fig. 3). The difference in the mean concentrations of TNFα protein was 145 pg/ml (95% confidence interval: 64-227) in the supernatant obtained from BAM challenged with *lkt* $^-$ compared to wt strain.

3. LKT-deficient *M. haemolytica* induced higher cytokine mRNA expression in BAM

A. Marked differences in the mRNA expression of various cytokines were observed in *lkt*$^-$ -challenged BAM compared to wt strain challenged BAM ($p = 0.020$). Both *lkt*$^-$ and wt challenged BAM had significant increases in TNFα mRNA expression ($p = 0.002$ and 0.018, respectively (Fig. 4). No significant difference ($p = 0.219$) in TNFα mRNA expression was observed between *lkt*$^-$challenged BAM and wt- challenged BAM. A bimodal expression of the TNFα mRNA was seen in both wt and *lkt*$^-$challenged BAM. An increased mRNA expression, in both *lkt*$^-$ - and wt-challenged BAM, was seen at 1 hour (mean = 28.8; SE = 0.8), which peaked at 2 hours (mean = 57.0; SE = 1.2), declined at 6 hours and peaked again at 12 hours (mean = 82.5; SE = 0.8) (Fig. 4). The TNFα

mRNA expression in BAM challenged with both wt and *lkt*⁻ strains returned near basal level by 24 hours (Fig. 4).

B. IL-1ß mRNA expression- Both *lkt*⁻ and wt challenged BAM had significant increases in IL-1β mRNA expression ($p = 0.006$ and 0.035, respectively). In *lkt*⁻-challenged BAM, an average of 7.4-fold increased (SE = 0.6) mRNA expression was seen at 2 hours followed by a decline by 6 hours and subsequent rapid increase at 12 hours (mean = 24.9; SE = 1.3). A similar but less substantial pattern of increase in mRNA expression was observed in wt-challenged BAM. The fold increase peaked (mean = 7.8; SE = 0.4) by 12 hours (Fig. 5). No significant difference in IL-1ß mRNA expression was observed between wt and *lkt*⁻ challenged BAM ($p = 0.408$).

C. IL-8 mRNA- Both *lkt*⁻ and wt challenged BAM had significant increases in IL-8 mRNA expression ($p < 0.001$). A biphasic expression reaching a peak by 2 hours (mean = 10.5; SE = 0.5) and again by 12 hours (mean = 24.0; SE = 1.3) was observed in *lkt*⁻ challenged BAM. In contrast, a steady and less substantial increase expression of IL- 8 mRNA was seen in wt-challenged BAM (Fig 6). No significant difference in IL-1ß mRNA expression was observed between wt and *lkt*⁻ challenged BAM ($p = 0.333$).

D. The pattern and fold-increase in IL-10 mRNA expression were similar between *lkt*⁻ and wt-challenged BAM ($p = 0.512$). Both *lkt*⁻ and wt-challenged BAM had significant increases in IL-10 mRNA ($p < 0.001$), which peaked at 2 hours with a mean fold increase of 18.3-fold (SE = 0.1) and 7.4-fold (SE = 0.5), respectively (Fig. 7). Somewhat similar unimodal kinetics of IL-12 mRNA was observed. Nearly no increase in IL-12 mRNA expression was observed besides a 37.5-fold (SE = 1.3) increased expression at 8 hours in *lkt*⁻-challenged BAM. No significant increase ($p = 0.431$)) in IL-12 mRNA expression was observed during the 24-hour period in wt-challenged BAM (Fig. 8). Similar to the patterns of IL-10 and IL-12 cytokines, a single peak of 14.2-fold (SE = 0.9) increase in IL-6 mRNA expression was observed at 2 hours in *lkt*⁻-challenged BAM. Again, no significant increase ($p = 0.128$) in IL-6 mRNA expression was observed in wt-challenged BAM during the 24-hour period (Fig 9).

4. *M. haemolytica* LKT causes cell cytotoxicity

BAM challenged with wt strain exhibited, in average, 43% more cytotoxicity than *lkt*$^-$ -challenged BAM ($p < 0.01$) indicating cell-cytotoxicity plays a role in decreasing cytokine production by wt-challenged BAM compared to *lkt*$^-$ -challenged BAM. 5-20% cytotoxicity was observed in *lkt*$^-$-challenged BAM. Five percent (SE = 1.3%) and 45% (SE = 7.8%) cytotoxicity was observed in *lkt*$^-$- and wt-challenged BAM after 1 hour incubation, respectively. After 2 hours of incubation, 19% (SE = 3.9%) and 57% (SE = 2.6%) cytotoxicity was observed in *lkt*$^-$- and wt-challenged BAM, respectively. The cytotoxicity of BAM increased to 71% (SE = 7.0%) and 20% (SE = 5.0%) following 6 hour incubation with wt and *lkt*$^-$strain (Fig. 10). On average, wt-challenged BAM exhibited 43% more cytotoxicity than *lkt*$^-$-challenged BAM ($p < 0.001$).

Discussion

Various virulence factors are associated with *M. haemolytica,* among these LKT and LPS are two critical virulence factors that play significant roles in the pathogenesis of shipping fever [3].[2] Though, the virulence of these two factors is well-documented through multiple *in vitro* studies using purified LPS and/or LKT, studies using whole bacteria containing all virulence factors, are limited [7, 26, 27]. We challenged BAM with the wt and *lkt*$^-$ *M. haemolytica* at the concentration of 1 bacterium per macrophage. The effects on macrophage function were measured by quantifying cytokine response using ELISA and RT-PCR. The results presented in this study demonstrate that *M. haemolytica,* presumably, through the use of multiple virulence factors has a powerful and selective effect on the induction of cytokine mRNA synthesis by BAM.

Bovine alveolar macrophages when challenged with *lkt*$^-$ strain produced significantly increased quantities of TNFα and IL-10 proteins compared to BAM challenged with the wt strain. A similar trend in mRNA expression of proinflammatory (TNFα, IL-8, and IL-1ß) and regulatory cytokines (IL-10 and IL-12) was observed. Though, an overall effect including all cytokines together was statistically significant between the two groups, no significant difference was observed between two groups for individual cytokines. The

cause for this difference in mRNA expression was, most-likely, cell cytotoxicity induced by LKT because BAM challenged with the wt strain at the concentration of 1 bacterium per cell exhibited, on average, 43% more cytotoxicity than *lkt*$^-$-challenged BAM. LKT is involved in down-regulation of MHC-II expression on bovine monocytes [28]; however, it is not known whether it exhibits similar effect on cytokines which may be an alternative explanation for decreased cytokine expression in *lkt*$^-$- challenged BAM. LPS can induce apoptosis and can enhance the cytotoxic effects of LKT[29]. It seems highly unlikely that the difference in cytokine mRNA expression we demonstrated could be related to altered LPS composition in *lkt*$^-$ strain. Murphy et al., did not observe any alteration in LPS in this *lkt*$^-$ strain which suggest that altered LPS composition in this strain is not responsible for the difference in observed cytokine mRNA expression pattern[30]. The results presented in this study also support previous findings that multiple cytokines are involved and in the pathogenesis of shipping fever [13].

In most mammalian models, TNFα, IL-1ß, and IL-8 are central components of a complex cytokine network that initiates, amplifies, and sustains the inflammatory response in tissue[16]. The results obtained from our research support previous studies in that multiple cytokines contribute to the pneumonia induced by *M. haemolytica* [16, 17, 31]. Tumor necrosis factor-alpha is a proinflammatory cytokine and its expression in the early stages of pneumonia induced by *M. haemolytica* has been reported[13, 31, 33]. On the other hand, IL-10 is a regulatory anti-inflammatory cytokine and is expected to play a role in resolving stage of pneumonia. The principal routine function of IL-10 appears to be to limit and ultimately terminate inflammatory responses. It is recognized for its ability to inhibit activation and effector function of T cells, monocytes, and macrophages and to regulate growth and/or differentiation of B cells, NK cells, cytotoxic and helper T cells, granulocytes, and endothelial cells[33]. We observed a significant amount of TNFα protein in the supernatant obtained from BAM soon after challenge with both *lkt*$^-$ and wt strains whereas minimal IL-10 protein was detected in the supernatant during that phase. However, the highest concentration of IL-10 protein was observed at 12 hours after challenge, which was followed by a decline in the TNFα protein and IL-1ß, IL-6, and IL-12 mRNA suggesting that cytokine IL-10 may be involved in regulation of these

cytokines. Kinetics of cytokine IL-10 and IL-12 mRNA expression in BAM challenged with *M. haemolytica* have not been previously reported. Interestingly, expression of IL-12 mRNA was observed in BAM starting 6 hours after challenge with *lkt*$^{-}$ strain but only mild expression was observed in BAM challenged with wt strain suggesting that in addition to the induction of cell cytotoxicity, LKT may be involved in down regulation of IL-12 and other cytokine mRNA expression.

Morphologically, *M. haemolytica* induced pneumonia is characterized by severe pulmonary necrosis which is attributed to the influx of neutrophil and subsequent release of reactive oxygen and nitrogen radicals and lysosomal enzymes due to cell cytotoxicity induced by LKT[7]. IL-8 is a major chemoattractant cytokine for neutrophils which can also activate these cells to undergo oxidative burst. As expected, we demonstrated mRNA expression of IL-8 in BAM challenged with both *lkt*$^{--}$ and wt strain indicating LPS induced production of IL- 8. A similar pattern for IL-8 mRNA expression in BAM challenged with purified LKT and LPS has been reported earlier by Lafleur et al., 2001[13]. Taken together, these results support the previous study where an increased quantities of IL-8 was observed in *M. haemolytica* induced pulmonary lesions suggesting that IL-8 could be a major neutrophilic chemoattractant cytokine for neutrophils in BPP[34].

Both LPS and LKT molecules of *M. haemolytica* can activate bovine leukocytes to produce proinflammatory cytokines[31]. Although, LKT is considered to play a critical role in inducing the typical pulmonary damage seen in BPP, we demonstrated expression of proinflammatory cytokines in both *lkt*$^{-}$ and wt-challenged BAM. These results in conjunction with similar results obtained from other studies suggest that LPS and other virulence factors play an important role in inducing pulmonary pathology [31, 35]. This speculation is further supported by the study conducted by Whitely et al., (1990) who demonstrated LPS localization within the cytoplasm of neutrophils, alveolar macrophages, endothelial cells, pulmonary intravascular macrophages, and on epithelial cell surfaces whereas LKT was associated with cell membranes of degenerating inflammatory cells located in the alveolus [36].

In an experimentally infected calf model of pneumonic pasteurellosis Highlander et al., (2000) and Tatum et al., (1998) demonstrated reduction in the clinical and lung lesion scores by using *lkt*¯ mutant *M. haemolytica.* These mutants were created by mutation on *lktA* and *lktC* genes of LKT. *lktC* encodes a transacylase that post-translationally modifies the inactive pro-LKT A to biologically active LKT whereas *lktA* encodes the inactive pro-LKT A. Reduced pulmonary lesions characterized by a reduction in necrosis and number of degenerate neutrophils were observed histologically[14, 15]. Unfortunately, cytokine evaluation was not performed in any study [4, 15]. The molecular basis underlying the difference in the lung lesions and attenuation of the virulence in *lkt*⁻ mutated *M. haemolytica* could be associated with the increased expression of cytokine IL-10 in *lkt*⁻ challenged BAM and subsequent reduction in the concentration of cytokines in lungs of calves challenged with *lkt*⁻ mutated *M. haemolytica* compared to wt-strain. It is also possible that the *lkt*⁻ challenged BAM survive long enough to reduce the inflammation through the effects mediated by elevated IL-10. IL- 10 is a regulatory cytokine that can be produced by macrophages, lymphocytes, dendritic cells and epithelial cells. It has predominantly antiinflammatory and immunosuppressive down-regulating effects on both innate and adaptive immune response. It suppresses the antigen-presenting functions of macrophages by down regulating MHC II, costimulatory molecules and inhibits IL- 12 production, which in turn inhibit the effective generation and/or maintenance of antigen-specific Th1 cells [33, 37].

In conclusion, we observed an overall increased mRNA expression of various cytokines TNFα, IL-1ß, IL-8, IL-10 and IL-12 when BAM were challenged with *lkt*⁻ strain compared to the wt strain. LKT induced cell cytotoxicity was observed in BAM which suggest that the difference in cytokine mRNA expression is due to cell cytotoxicity and cytokine expression is mediated by LPS or other virulence factors. The results presented in this study support previous findings that multiple cytokines are involved and that virulence factors other than LKT play important role in the pathogenesis of shipping fever [13].

References

1. Whiteley LO, Maheswaran SK, Weiss DJ et al. Pasteurella haemolytica A1 and bovine respiratory disease: pathogenesis. J Vet Intern Med 1992;6(1):11-22.

2. Rice JA, Carrasco-Medina L, Hodgins DC et al. Mannheimia haemolytica and bovine respiratory disease. Anim Health Res Rev 2007;8(2):117-28.

3. Confer AW, Panciera RJ, Clinkenbeard KD et al. Molecular aspects of virulence of Pasteurella haemolytica. Can J Vet Res 1990;54 Suppl:S48-52.

4. Highlander SK. Molecular genetic analysis of virulence in Mannheimia (pasteurella) haemolytica. Front Biosci 2001;6:D1128-50.

5. Hsuan SL, Kannan MS, Jeyaseelan S et al. Pasteurella haemolytica leukotoxin and endotoxin induced cytokine gene expression in bovine alveolar macrophages requires NF-kappaB activation and calcium elevation. Microb Pathog 1999;26(5):263-73.

6. Narayanan SK, Nagaraja TG, Chengappa MM et al. Leukotoxins of gram-negative bacteria. Vet Microbiol 2002;84(4):337-56.

7. Maheswaran SK, Weiss DJ, Kannan MS et al. Effects of Pasteurella haemolytica A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals. Vet Immunol Immunopathol 1992;33(1-2):51-68.

8. Clinkenbeard KD, Mosier DA, Confer AW. Effects of Pasteurella haemolytica leukotoxin on isolated bovine neutrophils. Toxicon 1989;27(7):797-804.

9. Atapattu DN, Czuprynski CJ. Mannheimia haemolytica leukotoxin induces apoptosis of bovine lymphoblastoid cells (BL-3) via a caspase-9-dependent mitochondrial pathway. Infect Immun 2005;73(9):5504-13.

10. Thumbikat P, Dileepan T, Kannan MS et al. Characterization of Mannheimia (Pasteurella) haemolytica leukotoxin interaction with bovine alveolar macrophage beta2 integrins. Vet Res 2005;36(5-6):771-86.

11. Marcatili A, D'Isanto M, Galdiero M et al. Role of Pasteurella multocida, Pasteurella haemolytica and Salmonella typhimurium porins on inducible nitric oxide release by murine macrophages. Res Microbiol 2000;151(3):217-28.

12. Stevens P, Czuprynski C. Dissociation of cytolysis and monokine release by bovine mononuclear phagocytes incubated with Pasteurella haemolytica partially purified leukotoxin and lipopolysaccharide. Can J Vet Res 1995;59(2):110-7.

13. Lafleur RL, Malazdrewich C, Jeyaseelan S et al. Lipopolysaccharide enhances cytolysis and inflammatory cytokine induction in bovine alveolar macrophages exposed to Pasteurella (Mannheimia) haemolytica leukotoxin. Microb Pathog 2001;30(6):347-57.

14. Highlander SK, Fedorova ND, Dusek DM et al. Inactivation of Pasteurella (Mannheimia) haemolytica leukotoxin causes partial attenuation of virulence in a calf challenge model. Infect Immun 2000;68(7):3916-22.

15. Tatum FM, Briggs RE, Sreevatsan SS et al. Construction of an isogenic leukotoxin deletion mutant of Pasteurella haemolytica serotype 1: characterization and virulence. Microb Pathog 1998;24(1):37-46.

16. Malazdrewich C, Ames TR, Abrahamsen MS et al. Pulmonary expression of tumor necrosis factor alpha, interleukin-1 beta, and interleukin-8 in the acute phase of bovine pneumonic pasteurellosis. Vet Pathol 2001;38(3):297-310.

17. Malazdrewich C, Thumbikat P, Abrahamsen MS et al. Pharmacological inhibition of Mannheimia haemolytica lipopolysaccharide and leukotoxin-induced cytokine expression in bovine alveolar macrophages. Microb Pathog 2004;36(3):159-69.

18. Murphy GL, Whitworth LC. Construction of isogenic mutants of Pasteurella haemolytica by allelic replacement. Gene 1994;148(1):101-5.

19. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141(2):471-501.

20. Confer AW, Ayalew S, Panciera RJ et al. Recombinant Mannheimia haemolytica serotype 1 outer membrane protein PlpE enhances commercial M. haemolytica vaccine-induced resistance against serotype 6 challenge. Vaccine 2006;24(13):2248-55.

21. Rottman JB, Tompkins WA, Tompkins MB. A reverse transcription-quantitative competitive polymerase chain reaction (RT-qcPCR) technique to measure cytokine gene expression in domestic mammals. Vet Pathol 1996;33(2):242-8.

22. Brown WC, Zhu D, Shkap V et al. The repertoire of Anaplasma marginale antigens recognized by CD4(+) T-lymphocyte clones from protectively immunized cattle is diverse and includes major surface protein 2 (MSP-2) and MSP-3. Infect Immun 1998;66(11):5414-22.

23. Hurvich CM, Tsai CL. Model selection for extended quasi-likelihood models in small samples. Biometrics 1995;51(3):1077-84.

24. Kenward MG, Roger JH. Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 1997;53(3):983-97.

25. Kwong LS, Hope JC, Thom ML et al. Development of an ELISA for bovine IL-10. Vet Immunol Immunopathol 2002;85(3-4):213-23.

26. Jeyaseelan S, Kannan MS, Hsuan SL et al. Pasteurella (Mannheimia) haemolytica leukotoxin-induced cytolysis of bovine leukocytes: role of arachidonic acid and its regulation. Microb Pathog 2001;30(2):59-69.

27. Thumbikat P, Briggs RE, Kannan MS et al. Biological effects of two genetically defined leukotoxin mutants of Mannheimia haemolytica. Microb Pathog 2003;34(5):217-26.

28. Hughes HP, Campos M, McDougall L et al. Regulation of major histocompatibility complex class II expression by Pasteurella haemolytica leukotoxin. Infect Immun 1994;62(5):1609-15.

29. Li J, Clinkenbeard KD. Lipopolysaccharide complexes with Pasteurella haemolytica leukotoxin. Infect Immun 1999;67(6):2920-7.

30. Murphy GL, Whitworth LC, Clinkenbeard KD et al. Hemolytic activity of the Pasteurella haemolytica leukotoxin. Infect Immun 1995;63(8):3209-12.

31. Jeyaseelan S, Sreevatsan S, Maheswaran SK. Role of Mannheimia haemolytica leukotoxin in the pathogenesis of bovine pneumonic pasteurellosis. Anim Health Res Rev 2002;3(2):69-82.

32. Morsey MA, Van-Kessel AG, Mori Y et al. Cytokine profiles following interaction between bovine alveolar macrophages and Pasteurella haemolytica. Microb Pathog 1999;26(6):325-31.

33. Moore KW, de Waal Malefyt R, Coffman RL et al. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683-765.

34. Caswell JL, Middleton DM, Sorden SD et al. Expression of the neutrophil chemoattractant interleukin-8 in the lesions of bovine pneumonic pasteurellosis. Vet Pathol 1998;35(2):124-31.

35. Paulsen DB, Confer AW, Clinkenbeard KD et al. Pasteurella haemolytica lipopolysaccharide-induced cytotoxicity in bovine pulmonary artery endothelial monolayers: inhibition by indomethacin. Vet Pathol 1995;32(2):173-83.

36. Whiteley LO, Maheswaran SK, Weiss DJ et al. Immunohistochemical localization of Pasteurella haemolytica A1-derived endotoxin, leukotoxin, and capsular polysaccharide in experimental bovine Pasteurella pneumonia. Vet Pathol 1990;27(3):150-61.

37. Nylen S, Sacks D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol 2007;28(9):378-84.

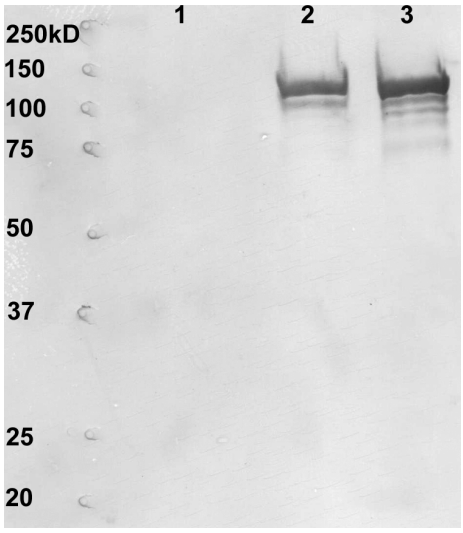

Fig. 1. Western blot of cell culture supernatant obtained from *lkt*$^{-}$- (lane 1), wt - (lane 2) and previously confirmed reference wt- (lane 3) *M. haemolytica* probed with anti-leukotoxin monoclonal antibody (MM 605). Lanes 2 and 3 contain 102 kDa *LKT* band along with the disintegrated fragments of *LKT* no such band is visible in lane 1.

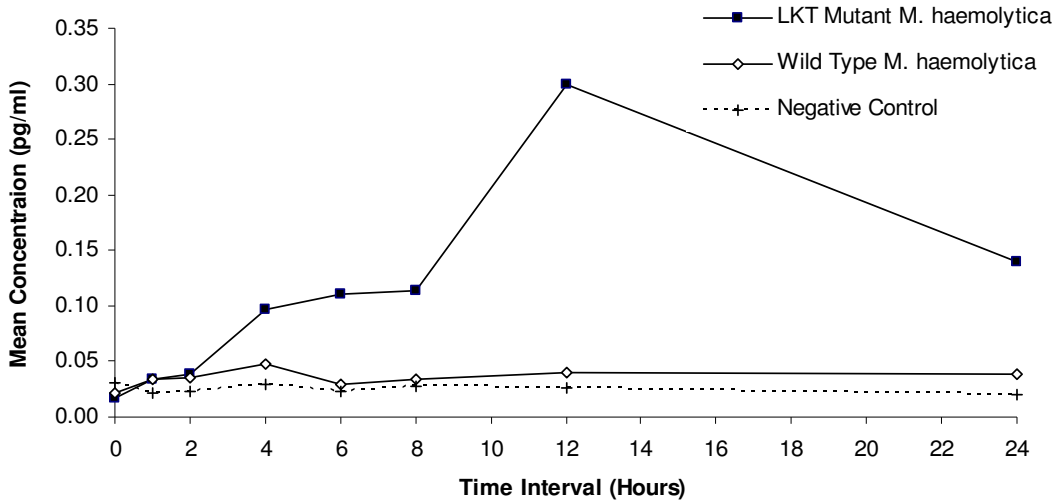

Fig.2. IL-10 protein secretion by BAM. BAM (5 x 10^6/ well) were cultured in 6-well plates in 2 ml RPMI and stimulated with wild type (wt) and *lkt*$^-$- *M. haemolytica* at 1:1 cell ratio. Tissue culture supernatant was harvested at the indicated time points and assayed for the presence of IL 10 protein using ELISA. Data are the means of samples obtained from 3 different animals. **: $p < 0.01$ comparing *lkt*$^-$ strain to wt strain at a particular time point.

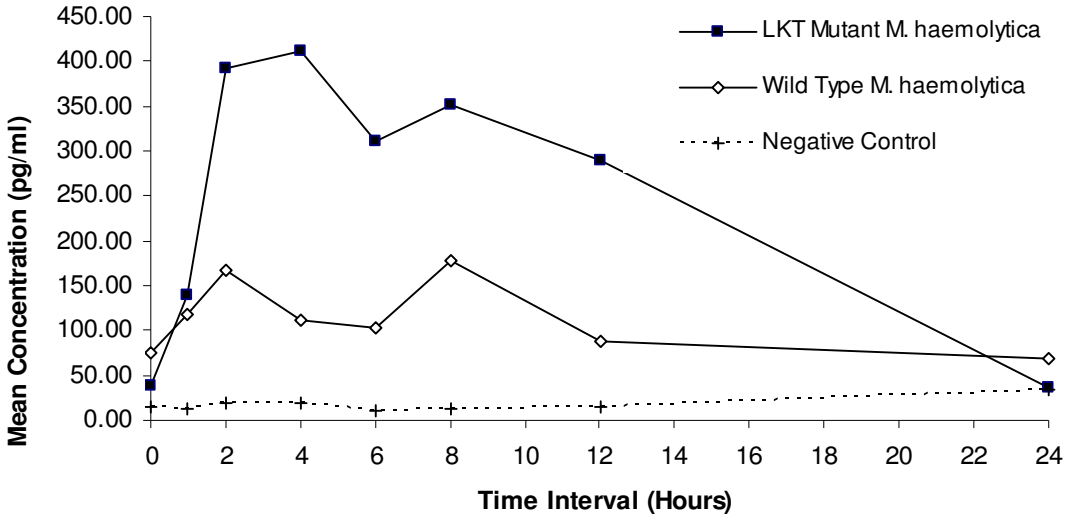

Fig.3. TNFα protein secretion by BAM. BAM (5 x 10^6/ well) were cultured in 6-well plates in 2 ml RPMI and stimulated with wild type (wt) and *lkt*$^-$- *M. haemolytica* at 1:1 cell ratio. Tissue culture supernatant was harvested at the indicated time points and assayed for the presence of TNFα protein using ELISA. Data are the means of samples obtained from 3 different animals. **: $p<0.01$ comparing *lkt*$^-$ strain to wt strain at a particular time point

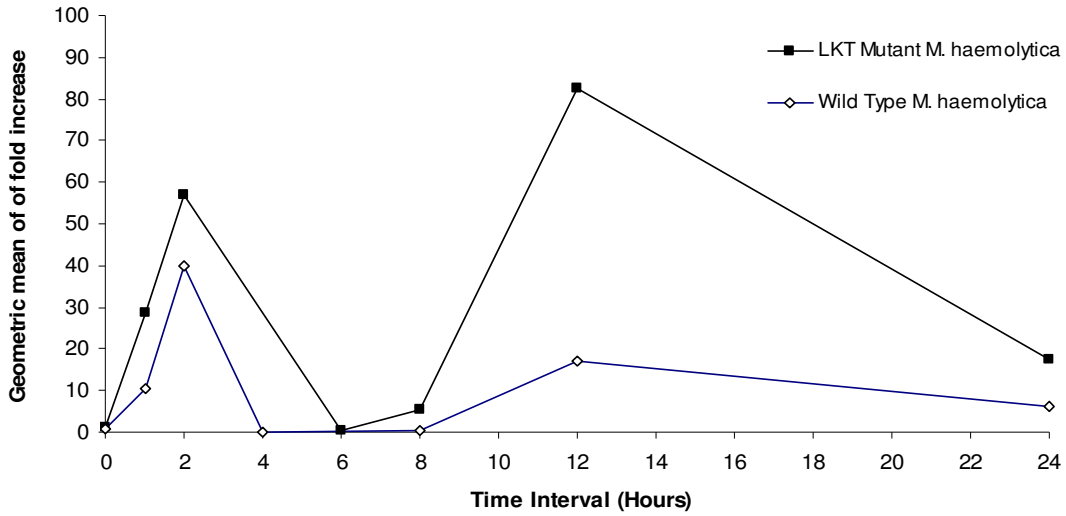

Fig. 4. Kinetics of TNFα mRNA production by BAM. BAM (5 x 10^6/ well) were cultured in 6-well plates in 2 ml RPMI and stimulated with wild type and *lkt*$^-$- *M. haemolytica* at 1:1 cell ratio. BAM were harvested at the indicated time points and assayed for the presence of TNFα mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase. Data are the geometric means of samples obtained from 4 different animals.

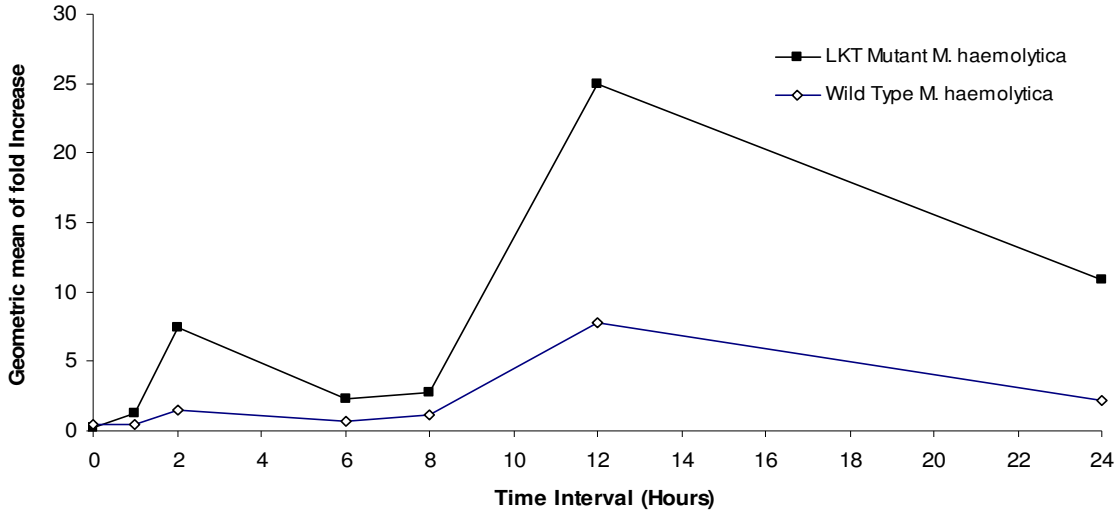

Fig. 5. Kinetics of IL-1ß mRNA production by BAM. BAM (5 x 10^6/ well) were cultured in 6 well plate in 2 ml RPMI and stimulated with wt and *lkt*- *M. haemolytica* at 1:1 cell ratio. BAM were harvested at the indicated time points and assayed for the presence of TNFα mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase. Data are the means ± SEM of samples obtained from 3 different animals.

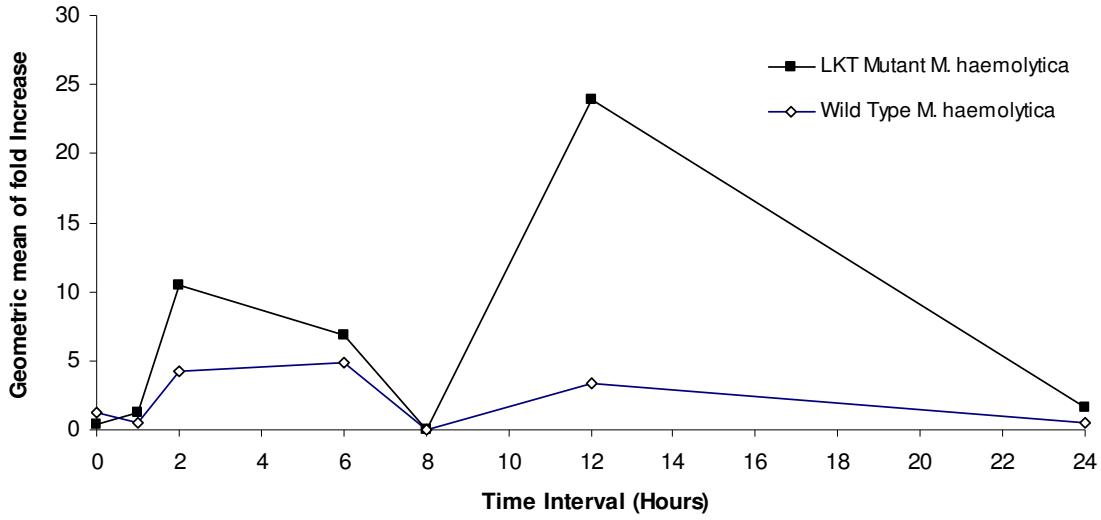

Fig. 6. Kinetics of IL 8 mRNA production by BAM. BAM (5 x 10^6/ well) were cultured in 6-well plates in 2 ml RPMI and stimulated with wild type and *lkt*$^-$ - *M. haemolytica* at 1:1 cell ratio. BAM were harvested at the indicated time points and assayed for the presence of IL 8 mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase. Data are the geometric means of samples obtained from 3 different animals.

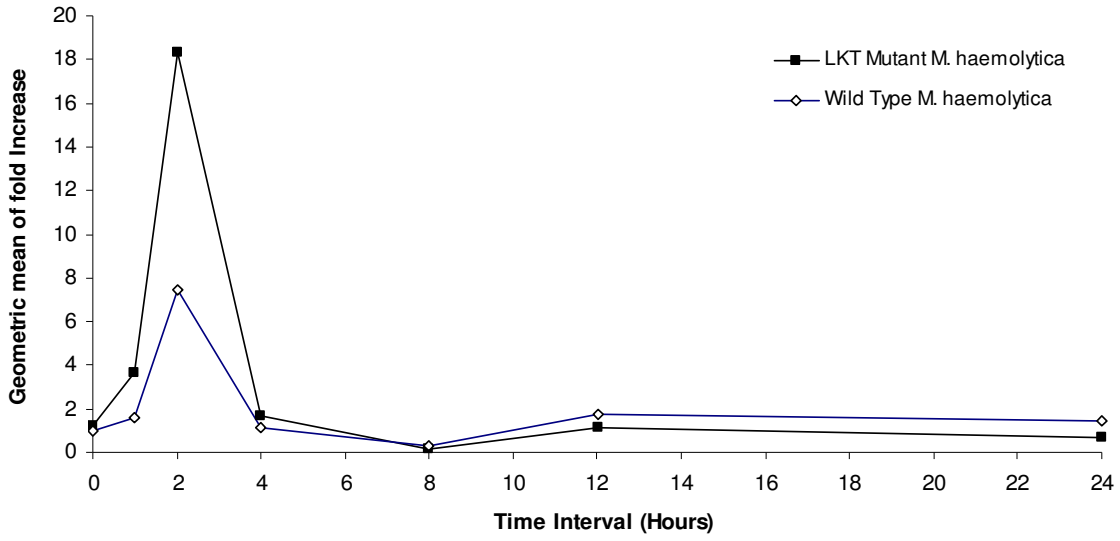

Fig. 7. Kinetics of IL 10 mRNA production by BAM. BAM (5 x 10^6/ well) were cultured in 6 well plate in 2 ml RPMI and stimulated with wt and *lkt*$^-$- *M. haemolytica* at 1:1 cell ratio. BAM were harvested at the indicated time points and assayed for the presence of TNFα mRNA using RT-PCR. MRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase. Data are the geometric means of samples obtained from 3 different animals.

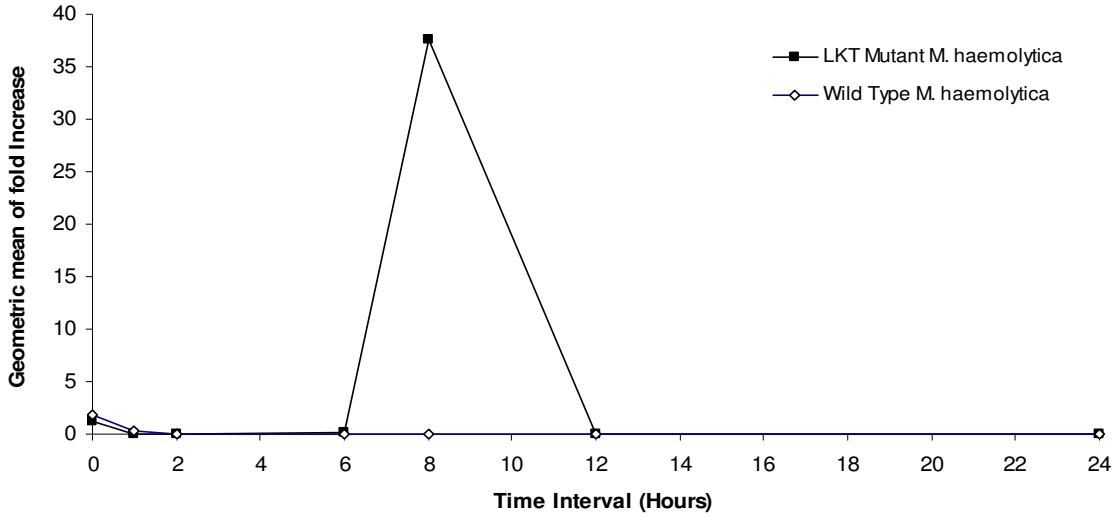

Fig.8. Kinetics of IL 12 mRNA production by BAM. BAM (5 x 10^6/ well) were cultured in 6 well plate in 2 ml RPMI and stimulated with wt and *lkt*$^-$- *M. haemolytica* at 1:1 cell ratio. BAM were harvested at the indicated time points and assayed for the presence of TNFα mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase. Data are the geometric means of samples obtained from 3 different animals.

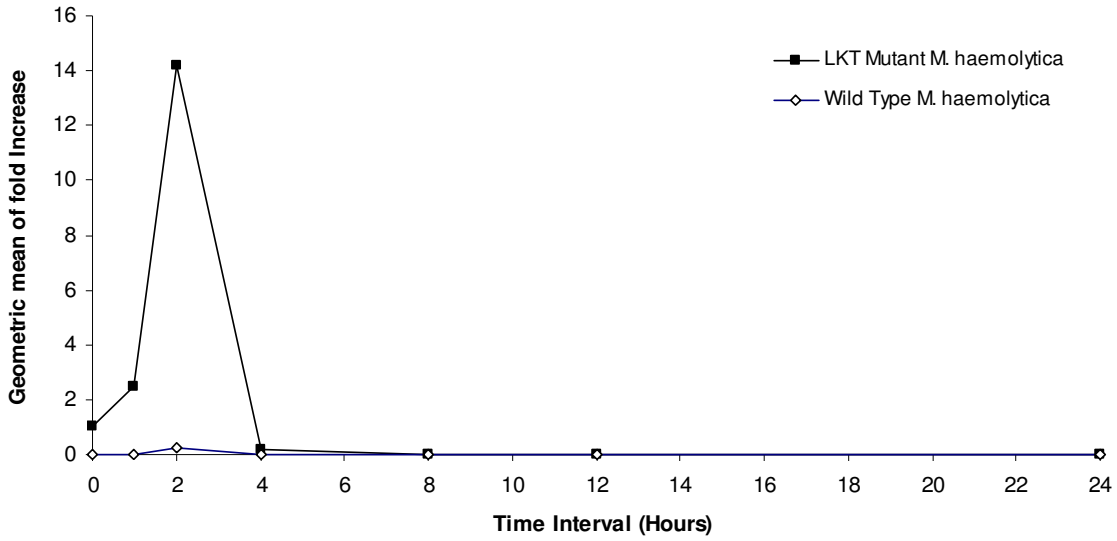

Fig. 9. Kinetics of IL 6 mRNA production by BAM. BAM (5 x 10^6/ well) were cultured in 6 well plate in 2 ml RPMI and challenged with wt and *lkt*$^-$- *M. haemolytica* at 1:1 cell ratio. BAM were harvested at the indicated time points and assayed for the presence of TNFα mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase. Data are the geometric means of samples obtained from 3 different animals.

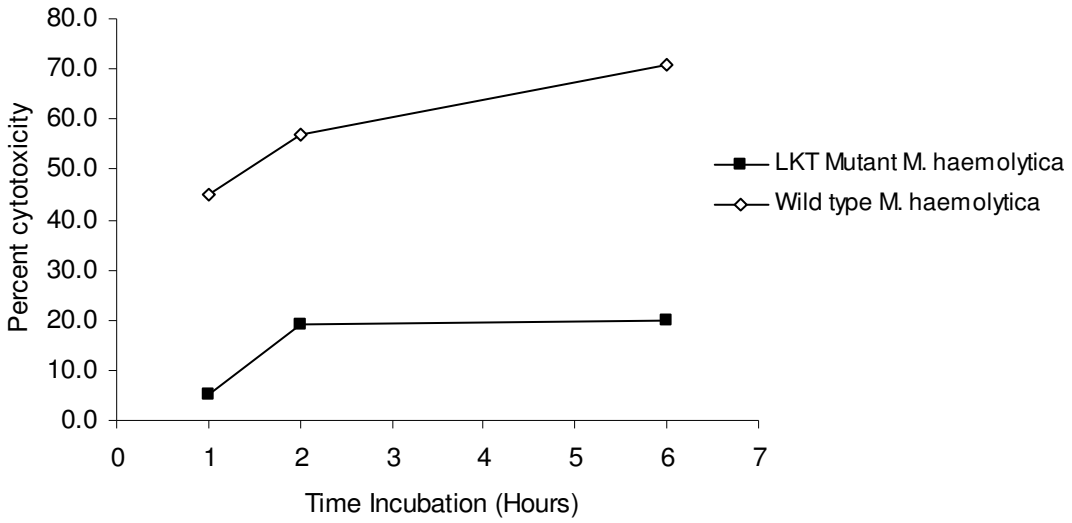

Fig. 10. Percent cytotoxicity for bovine alveolar macrophages challenged with *lkt*$^{-}$- and wild type (wt)-strains of *M. haemolytica*. Data are the means of the samples obtained from 3 different animals. Cytotoxicity was quantified using the Thiazolyl Blue Tetrazolium Bromide assay at 1, 2, and 6 hours after incubation. An error reading for wt-challenged group at 6 hour was excluded from the data analysis. **: $p < 0.01$.

CHAPTER IV

Cytokine evaluation of inflammatory cells in calves challenged with the wild type and leukotoxin deficient *Mannheimia haemolytica*

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Leukotoxin (LKT) and lipopolysaccharide (LPS) are important virulence factors associated with *Mannheimia haemolytica*. LKT is an exotoxin specific for ruminant leukocytes bearing β2 integrin receptor and exhibits dose-dependent effects on bovine leukocytes ranging from activation to cell cytotoxicity. Role of LKT in the pathogenesis of shipping fever is documented by multiple *in vitro* studies and limited *in vivo* studies. The results of these *in vivo* experiments are diverse. The objective of this study is to document the role of LKT in modulating cytokine response in lungs of calves challenged with LKT deficient (*lkt*$^-$) and wild-type (wt) *M. haemolytica*. Thirty-six calves, seronegative to LKT and *M. haemolytica* whole cell antigen were divided among three groups (I-III). Group I and Group II calves were challenged intratracheally with 25 ml PBS containing 0.44 x 10^9 cfu/ml *lkt*$^-$ and 25 ml PBS containing 0.31 x 10^9 cfu/ml of wt *M. haemolytica*, respectively, whereas Group III calves were challenged intratracheally with 25 ml sterile PBS. The effects on leukocytes function were measured by quantifying the cytokine IL-1β, IL-8, IL-10, IL-12 and TNFα response using ELISA and real-time RT-PCR. Clinically, calves challenged with wt-strain exhibited higher mean clinical score and one death compared to calves challenged with *lkt*$^-$-strain. The composition of bronchioalveolar lavage fluid was variable between the groups. A statistically significant difference in the expression level of cytokines was not observed between the groups challenged with *lkt*$^-$ and wt-strain. By the end of the experiment, on day 6 post-inoculation, both Groups I and II calves seroconverted to *M. haemolytica*. These results

suggest that multiple cytokines are involved and that virulence factors other than LKT play important role in the pathogenesis of shipping fever.

Key words: cytokine, leukotoxin, real-time RT-PCR, *Mannheimia haemolytica*, shipping fever

Introduction

Mannheimia haemolytica is the major cause of fibrinous and necrotizing pleuropneumonia of cattle termed bovine pneumonic pasteurellosis (BPP) or shipping fever. Several virulence factors of *M. haemolytica* promote host-pathogen interactions by assisting the bacterium to colonize cattle lungs and contribute to the development of pneumonia. Leukotoxin (LKT) and lipopolysaccharide (LPS) are critically important virulence factors, and their roles in the pathogenesis of shipping fever are well documented[1].Other potential virulence factors whose pathogenic roles are less well documented include capsule, fimbriae, iron-regulated proteins, outer membrane proteins, neuraminidase, and a neutral glycoprotease[1, 2].

Most of the knowledge that has been generated about the role of LKT and LPS in the pathogenesis of shipping fever comes from multiple *in vitro* studies using purified LKT alone or in combination with LPS[3, 4]. Because LKT and LPS exhibit synergism, their pathological roles are better understood when used in conjunction with each other[5]. *In vivo* studies using the whole bacterial approach are limited. Only two studies with characterized LKT mutations have been conducted so far and the results obtained from these studies are diverse [6, 7]. Highlander et al., (2000) and Tatum et al., (1998) demonstrated reduction in the clinical and lung lesion scores by using *lkt*$^{-}$ mutant compared to wt-strain of *M. haemolytica.* Reduced pulmonary lesions characterized by a reduction in necrosis and number of degenerate neutrophils were observed histologically[6, 7]. The molecular basis underlying the difference in the lung lesions and attenuation of the virulence in *lkt*$^{-}$ mutated *M. haemolytica* is purely speculative.

A growing body of evidence implicates inflammatory cytokines in the pathogenesis of BPM[5, 8]. Therefore, a comprehensive understanding of cytokines expression would enable precise assessment of the roles cytokines play in *M. haemolytica*-challenged calves. Heat-killed *M. haemolytica*, as well as purified LPS and LKT induce the expression of TNFα, IL-1β, and IL-8 genes and proteins in bovine

alveolar macrophages *in vitro*[5, 8] . We identified cytokines TNFα, IL-1ß, and IL-8 because these are pleiotropic early response proinflammatory molecules produced by a variety of cells and play significant roles in innate immunity[8]. The cytokine IL-12 was selected for the measurement because it is involved principally in regulating-adaptive immune response. The principal routine function of IL-10 appears to be to limit and ultimately terminate inflammatory responses[9].

In the present study, we investigated the role of LKT in modulating cytokine gene and protein expression in bovine inflammatory cells obtained from the lungs of calves challenged by an isogenic LKT-deficient (*lkt*$^-$) and wild type (wt) *M. haemolytica* strains. Our hypothesis was that the modulation of cytokine mRNA response observed in the acute and adaptive phase in inflammatory cells is modulated by LKT. The wt strain was isolated from a pneumonic bovine lung, and isogenic *lkt*$^-$ strain was prepared by an allelic replacement of the entire *lktA* and partial *lktC* gene with the beta-lactamase enzyme gene[10]. The western blot data supported that LKT was not produced by *lkt*$^-$ *M. haemolytica* (Singh et al., In press). Additionally, in contrast to the wt strain, no zone of peripheral hemolysis was associated with the colonies of the *lkt*$^-$-strain grown on sheep blood agar[10]. The objective of this study was to evaluate the effect of LKT on cytokine protein and mRNA expression in inflammatory cells obtained from lungs of calves following challenge with wt and *lkt*$^-$ strain *M. haemolytica*.

Material and methods

a. Bacterial inoculum

Both wt and *lkt*$^-$ *M. haemolytica* cultures had nearly identical growth curves and were prepared similarly. Briefly, bacteria were grown overnight on brain heart infusion (BHI) agar containing 5% sheep blood (Hardy Diagnostics, Santa Maria, CA) without and with ampicillin (10ug/ml) for wt and *lkt*$^-$ cultures, respectively. Multiple colonies were subsequently transferred to 150 ml of BHI broth and incubated for 6 hours at 37ºC at 70 oscillations per minute (opm). The broth culture was centrifuged twice at 8000 x g for 15 minutes. The bacterial pellet was washed with 10 ml PBS. After a second centrifugation,

the pellet was resuspended in RPMI media or PBS and the concentration was quantified using spectrophotometer (Pharmacia, Ultraspec 2000, Haverhill, MA). In our previous experiments, the OD of 0.72-0.74 at 650 nm corresponded to 10^9 cfu/ml[11]. The exact concentration of the viable bacterial suspension was subsequently confirmed by standard colony count at various dilutions on BHI blood agar which was 0.44 x 10^9 cfu/ml for *lkt*$^-$-strain and 0.31 x 10^9 cfu/ml for wt-strain.

b. Leukotoxin production and partial purification: Both wt and *lkt*$^-$ strains were grown on BHI supplemented with 5% sheep blood agar. A single colony was suspended in BHI broth and incubated overnight at 37ºC in a shaking incubator at 160 opm. The culture was subsequently resuspended in 1L RPMI 1640 (R7509 Sigma) and incubated at 37ºC at 160 opm. The culture was centrifuged at 3800 x g for 20 minutes. The supernatant obtained was filtered using 0.2 µ filters (Fisher 167-0020). The concentrate was repeatedly centrifuged (Millipore, Amicon ultra-15) at 2000 x g for 15 minutes. The culture supernatant containing semi purified LKT was aliquoted and stored at -86°C for further use in western blot.

c. Real-time RT-PCR: mRNA expression of multiple cytokines was determined by real time PCR. Briefly, the total RNA was extracted using the guanidine thiocyanate technique as described previously [12]and cDNA was generated by utilizing reverse transcriptase kit (QuantiTect Rev. Transcription Kit, Qiagen). RNAstat-60 (Tel-Test B, Friendswood, TX) was added to the pelleted cells and the cells lysed by pipetting up and down multiple times. The RNA was subsequently isolated according to the kits instructions, measured with spectrophotometer, and adjusted to 0.1 µg/µl with Diethyl pyrocarbonate (DEPC) treated water. Real-time RT-PCRs were performed in individual 500 µl Eppendorf tubes containing 12.5 µl of 1x TaqMan Universal PCR master mix, 1.5 µM each primer, 10 µl water, and 2·0 µl sample cDNA in a final volume of 26 µl. PCR amplification and detection were performed on an ABI Prism 7000 Sequence Detection System using the following cycling conditions: 50 °C for 2 min, 95 °C for

10 min and 40 cycles of 94 °C for 15 sec, 55°C for 30 sec, and 72 °C for 30 sec. All RT-PCRs were carried out in duplicate with appropriate controls run on each plate. Bovine beta actin mRNA was used as endogenous reference mRNA. The sequences of primers used in this study were published [13] previously. The geometric mean was calculated as [(ct value of a cytokine from a treated group – ct value of actin from that treated group) – (ct value a cytokine from untreated group – ct value of actin from untreated group).

d. Virulence study- Thirty-six 140 to 200 kg, mixed-gender, crossbreed calves were screened for the presence of anti-LKT and anti-*M. haemolytica* whole cell antigens at day -18 using ELISA as previously described . [17] Calves were selected as having minimal antibody concentration to *M. haemolytica*. Calves were transported to the test site 10 days prior to the experiment trial and were allowed to acclimatize. Calves were randomly assigned to Group I-III with 12 calves per group. Group I and Group II calves were challenged intratracheally with *lkt*$^-$ and wt *M. haemolytica*, respectively, whereas Group III calves served as control and were challenged with sterile PBS only. Calves from the different groups were housed in distantly located individual pens to avoid bacterial transfer by aerosol route. On day 0, each calf in Group I and Group II received a 25 ml of bacterial suspension in PBS containing 0.44 x 10^9 cfu/ml and 0.31 x 10^9 cfu/ml of *lkt*$^-$ and wt-strain, respectively, delivered intratracheally into the lungs using endotracheal tube and followed by a 25 ml PBS flush. Bronchoalveolar lavage (BAL) fluid was obtained from each calf on day -4, 1, 3 and 6 using 180 ml of sterile PBS via an endotracheal tube. The BAL was collected in 50 ml tubes containing 1% penicillin-streptomycin and amphotericin B (Cambrex, Walkersville, MD, USA) and was brought to laboratory on ice for cell isolation. The tubes containing BAL were centrifuged at 2000 rpm for 7 minutes. Five hundred-ul of supernatant was collected for ELISA. The remaining supernatant was discarded. The cell pallet was gently washed in 10 ml PBS and centrifuged again in 15 ml PBS at 500 x g for 7 minutes. The pellet obtained was resuspended in 1 ml RNA stat solution. Following challenge, the calves were clinically evaluated daily starting at day -1 until day 6 post-inoculation (PI). The clinical scoring system was based on a nonparametric scale[7]. A score of 0-4 points each was allotted

based on the following conditions: rectal temperature above 39C; depression; dyspnea or respiratory rate ≥ 60/min. The mean clinical score for each group was calculated.

e. Serum antibody responses to *M. haemolytica* antigens: Serum IgG antibodies to *M. haemolytica* serotype 1 LKT and whole cells (WC) antigens were determined by ELISA as previously described with minor modifications[14]. Briefly, LKT was prepared as described in section b. For obtaining WC, formalin-killed *M. haemolytica* was prepared from a washed 24 h culture by suspending cells in 0.4% formalinized saline at a concentration determined spectrophotometrically to be 1.850 at OD_{650}[15]. Wells of 96-well polystyrene EIA/RIA microtiter flat bottom high binding plates (COSTAR #9018 Corning Inc., Corning, NY, USA) were coated with LKT at 50 ng per well and WC. Primary antisera were diluted in blocking buffer solution consisting of 1% bovine serum albumin and 0.05% Tween-20 in PBS (pH 7.4) and assayed in triplicate. Bound antibodies were detected using peroxidase labeled goat monoclonal antibody to bovine IgG (H+L). Enzymatic activity was assayed using the color substrate ortho-phenylene diamine (Ameresco, 5348-50T). Optical densities of the wells were read at 650 nm. Antibody responses were expressed as nanograms of IgG binding compared to a standard immunoglobulin curve.

f. ELISA: A TNFα ELISA was performed according to the manufacturer's instructions (Endogen, Bovine TNF-alpha screening set, Rockford, IL, USA). ELISAs for other bovine cytokines were not available.

Results

1. LKT deficient *M. haemolytica* did not secrete LKT

The monoclonal antibody (MM 605) recognized the 102 kDa LKT band and associated breakdown bands in the supernatant obtained from the culture of wt *M. haemolytica,* whereas a similar band was not detected in the culture supernatant obtained from *lkt*$^-$ *M. haemolytica* (Singh et al., In press). The growth of the wt strain on 5% sheep blood agar was accompanied by a definite zone of hemolysis surrounding the colonies, whereas no zone of hemolysis was associated with the growth of *lkt*$^-$ strain (data not shown).

Additionally, bovine alveolar macrophages challenged with wt strain exhibited 55-75% cytotoxicity compared to challenge with the *lkt*¯strain (Singh et al., In press).

2. **Mean clinical score and rectal temperature**: Group I and Group II calves challenged with *lkt*⁻ and wt strain, respectively, had a higher mean clinical score compared to control group calves though out the study. However, the average mean clinical score during the first 48 hours was not different in the calves challenged with either *lkt*⁻ or wt strain (Fig. 1). Starting at day 3 until day 7 PI wt strain-challenged calves exhibited slightly higher mean clinical score ($p > 0.05$) compared to *lkt*⁻ strain-challenged calves (Fig. 1). In contrast to other calves of Group II, calf number 21 developed severe clinical signs of dyspnea, cough, depression, high rectal temperature and died within 72 hours of challenge. At necropsy, characteristic gross and microscopic lesions of fibrinonecrotic bronchopneumonia typical of *M. haemolytica* infection were observed. Large numbers of *M. haemolytica* and small numbers of *P. multocida* were cultured from the pneumonic lung of this calf. The calves of Group I and Group II had slightly higher mean ($p > 0.05$) rectal temperature starting 1 to 5 day PI when compared to calves of Group III (Fig. 2).

3. Antibody response of calves to LKT and whole cell antigens of *M. haemolytica*- All calves of Group I – Group III had low anti-LKT and anti-WC antibodies concentrations prior to challenge (days -18 and -4). On day 7 PI, elevated levels of anti-LKT and anti-WC antibodies were detected in the sera collected from the group I and II calves indicating seroconversion to *M. haemolytica* antigens (Fig. 3 and 4).

4. BAL composition: The BAL fluid composition in all calves prior to challenge (Day -4) consisted predominantly of alveolar macrophages (75%-89%) admixed with neutrophils (5.7% - 25%), lymphocytes (1.5%-44.9%) and rare eosinophils (Fig. 5-7). Following challenge on day 1, BAL fluid from Group III and Group II calves contained predominantly neutrophils (61.9% and 55.24%) admixed with macrophages (42.4% and 36.8%) and small population of lymphocytes (1.3% and 2.1%), and rare eosinophils (Fig. 5 and 6). In contrast, BAL fluid obtained from Group I calves constituted primarily of alveolar macrophages (96%) admixed with rare lymphocytes, neutrophils, and

eosinophils on day 1(Fig. 7). The composition of BAL fluid obtained on day 3 from Group I and Group II was 50.9% and 45.8% macrophages, 44.8% and 52.2% neutrophils admixed with 4.1% and 1.9% lymphocytes, respectively (Fig. 6 and 7). Composition of BAL fluid obtained from all groups on day 7 was comparable to pre-challenge composition with predominantly 53.7%-67.4% alveolar macrophages, 28.7% - 38.5% neutrophils, 3.02% -10.6% lymphocytes and rare eosinophils (0.09%-0.8%) (Fig. 5-7).

5. Quantification of TNFα in the BAL fluid using ELISA – On day -4, 70-80 pg/ml TNFα protein was present in the BAL fluid collected from the calves from Group I – III. A mild statistically insignificant ($p > 0.05$) increase to 90-95 pg/ml was observed in all groups 24 hours post challenge (day 1). On day 2, the concentration of TNFα decreased to 65-69 pg/ml in all groups and stayed in similar range on day 7 except for wt-challenged calves where a further reduction to 54 pg/ml was observed (Fig. 8)

6. Quantification of cytokine gene expression in cells obtained from BAL fluid

Real-time PCR was used to analyze mRNA expression of cytokines TNFα, IL-1, IL-8, IL-10, and IL-12 in the inflammatory cells obtained from BAL of calves challenged with *lkt*$^-$ and wt strain of *M. haemolytica.*

a. Proinflammatory cytokines- An increased mRNA expression of proinflammatory cytokines IL-1, IL-8, and TNFα was observed on day 1 PI compared to days -4, 3 and 6 PI except for increased IL-8 expression on day 3 in the inflammatory cells obtained from the lungs of Group II calves (Fig. 9). An increased baseline expression of IL-1 mRNA, compared to the remaining days 1, 3, and 6 PI, was observed on day -4 (Fig. 10). No statistically significant ($p > 0.05$) difference was found in the expression of aforementioned proinflammatory cytokines between Group I and Group II calves.

b. IL-10 and IL- 12 mRNA expression- We observed 7-fold increased expression of IL-10 mRNA on day 1 PI in cells obtained from the lungs of Group I calves compared to Group II calves. No significant difference ($p > 0.05$) was observed within the Group I and

Group II calves (Fig. 12). Unexpected increased expression of IL-12 mRNA was present at day -4 PI. This increased expression in Group I calves was followed a decline on day 1 and 3 with slight increased expression again on day 7 PI. In case of Group II calves, increased IL-12 expression was followed by a decline on day 1, 3 and 7 PI (Fig. 13). Again, no statistically significant differences ($p > 0.05$) were observed within Group I and Group II calves.

Discussion

Various virulence factors are associated with *M. haemolytica,* among these LKT and LPS are critical virulence factors that play significant roles in the pathogenesis of shipping fever.[2] The virulence of these factors is well-documented through multiple *in vitro* studies using purified LPS and/or LKT[5, 16, 17]. Unfortunately, studies using whole bacteria containing all virulence factors are limited. We intratracheally challenged calves with the *lkt*$^{-}$ and wt *M. haemolytica,* following challenge inflammatory cells were collected from the BAL obtained on day -4, 1, 3, and 7 PI. The effects on inflammatory cell functions were measured by quantifying cytokine response using ELISA and real-time RT-PCR. A statistically significant difference was not observed in the expression level of various cytokines between calves challenged with the wt and *lkt*$^{-}$ -strain.

Our findings are surprising in that LKT is considered the critical virulence factor in *M. haemolytica* induced pneumonia. In an experimentally infected calf model of pneumonic pasteurellosis Highlander et al., (2000) and Tatum et al., (1998) demonstrated reduction in the clinical and lung lesion scores by using *lkt*$^{--}$ mutant *M. haemolytica.* Isogenic LKT-deficient *M. haemolytica* was constructed by allelic replacement of *lktA,* produced mild clinical signs and decreased virulence in calves[7]. Lungs from calves challenged with wt-*M. haemolytica* contained numerous degenerate and streaming neutrophils (oat cells) in alveoli accompanied by fibrin and necrosis. In contrast, lungs from calves challenged with LKT A mutant had no degenerate or streaming neutrophils[7]. Highlander et al., (2000) created *lktC* mutant capable of

secreting inactive LKT by inserting bacteriophage P1 *loxP* site within the *lktC* open reading frame. This insertion caused frameshift mutation but did not alter the expression of other LKT genes including *lktA, lktB*, and *lktD.* Subsequently, a minimal reduction in the gross lung lesions characterized by reduced pleuritis and edema were observed in calves challenged intrathoracically with *lktC* mutant compared to wt-strain. Unfortunately, analysis of BAL composition and quantitative cytokine evaluation was not performed in these studies. Results obtained from our study may explain the lack of significant difference observed in lung lesions of calves challenged with LKT-deficient *M. haemolytica*. Alternatively, the lack of consensus on the ability of LKT-deficient *M. haemolytica* to reduce virulence *in vivo* could be due to the role of other virulence factors in particular LPS. LPS is known to produce similar cytokines response like LKT; therefore, it is possible that any potential difference in cytokine production by *lkt*$^{-}$ - and wt-strain in this study was overpowered by the effect of LPS[5]. Our findings suggest that although LKT is known to contribute to the pathogenesis of shipping fever, it is not the sole virulence factor and other virulence factors likely contribute significantly in the pathogenesis the disease. This presumption is supported by previous studies on another RTX exotoxin (*E. coli* hemolysin) which suggest a somewhat controversial role of this exotoxin in disease [18, 19]. Collectively, the results obtained from our study reinforce the role of other virulence factors in the pathogenesis of shipping fever in particular LPS.

Results from the studies by Highlander et al., (2000) and Tatum et al., (1998) indicate that while mutation on *lktA* created highly attenuated *M. haemolytica*, mutation of *lktC* only caused mild attenuation of virulence. LKT mutant created by Highlander et al., was generated by targeting *lktC* whereas LKT mutant created by Tatum et al., was constructed by allelic replacement of *lkt A*. *lktC* encodes a transacylase that post-translationally modifies the inactive pro-LKT A to biologically active LKT whereas *lktA* encodes the inactive pro-LKT A. The role of these mutations in the pathogenesis was further characterized and results suggest that LKT created by *lktA* and *lktC* mutation are not capable of activating neutrophils, inducing IL- 8 expression, and causing cytolysis[20]. The *lkt*$^{-}$ strain used in this study was generated by targeting both *lktA* and

lktC; therefore, it is highly unlikely that LKT secreted by *lkt*$^-$ strain was responsible for activating inflammatory cells to produce cytokines.

In our recent *in vitro* experiment we demonstrated that bovine alveolar macrophages (BAM) challenged with similar *lkt*$^-$-strain produced increased quantities of TNFα and IL-10 protein in the supernatant compared to wt-challenged BAM. Similarly, using real-time RT-PCR, increased mRNA expression of cytokines TNFα, IL-1ß, IL-8, IL-10 and IL-12 was observed in *lkt*$^-$ -challenged BAM compared to the wt-challenged BAM. This difference in the cytokine production was attributed to cell cytotoxicity induced by wt-strain[21]. Though, LKT-induced necrosis was observed histologically in the lungs of wt-challenged calf, it is possible that LPS stimulation of inflammatory cells overpowered the reduced cytokine expression due to LKT-induced leukocyte cytotoxicity [21].

The migration and activation of neutrophils within diseased lungs are regulated by a complex network of interactions between cytokines, leukocytes, vascular endothelium, cellular adhesion molecules, and soluble chemotactic factors[8]. Current evidence indicates that the proinflammatory cytokines, in particular, IL-8 play a central role in the initiation and orchestration of these interactions promoting neutrophil migration at the site of injury[22, 23]. Results obtained from *in vitro* studies using BAM indicate that biologically active LKT induce increased expression of IL-8 in these cells compared to mutant LKT[20]. The BAL fluid obtained from Group I calves on day 1 PI had less than 2% neutrophils whereas Groups II calves had predominantly neutrophils. This difference in the composition of BAL fluid may be due to the inability of *lkt*$^-$ strain to produce biologically active LKT. Decreased neutrophils in pulmonary parenchyma also corroborate the reduced pulmonary damage observed in previous *in vivo* studies using LKT mutant *M. haemolytica* [6, 7].

Interleukin-10 is a multifunctional cytokine with diverse effects on most inflammatory cell types. It has predominantly antiinflammatory and immunosuppressive down-

regulating effects on both innate and adaptive immune response and it ultimately terminates inflammatory response. It is recognized for its ability to inhibit activation and effector function of T cells, monocytes, and macrophages and to regulate growth and/or differentiation of B cells, NK cells, cytotoxic and helper T cells, granulocytes, and endothelial cells[9]. It suppresses the antigen-presenting functions of macrophages by down regulating MHC II, costimulatory molecules, and inhibits IL- 12 production, which in turn inhibit the effective generation and/or maintenance of antigen-specific Th1 cells[24]. We observed a marked expression of IL-10 mRNA on day 1 PI in cells obtained from group I calves (Fig. 12). If the difference exist in the level of cytokines generated *in vivo* by wt and *lkt*¯strain, then these differences could have been masked by upregulation of IL-10 in group I. Similarly, increased expression of IL-10 in *lkt*⁻ challenged BAM and subsequent reduction in the concentration of cytokines in lungs of calves challenged with *lkt*⁻ mutated *M. haemolytica* compared to wt-strain may be the molecular basis underlying the difference in the lung lesions and attenuation of the virulence in *lkt*⁻ mutated *M. haemolytica*[6, 7]. It is also possible that the *lkt*⁻ challenged BAM survive long enough to reduce the inflammation through the effects mediated by elevated IL-10. Alternatively, the lack of statistically significant differences in the cytokine mRNA expression in calves challenged with *lkt*¯ or wt-strain could be due to the dose of bacteria used to challenge calves. The dose used in this study selected from a previous study in order to demonstrate altered cytokine production by inflammatory cells obtained from lungs without inducing mortality[25]. In spite of no statistically significant differences in the cytokine production, the wt-challenged calves exhibited higher clinical score compared to *lkt*¯-challenged calves. One calf challenged with wt-strain died after 72 hours of inoculation whereas no mortality was observed in *lkt*⁻ -challenged calves. The necropsy on this calf revealed necrotizing bronchopneumonia typical of *M. haemolytica* and large numbers of *M. haemolytica* were cultured from the lung.

LKT ELISA revealed that calves were seronegative prior to challenge but only mild seroconversion to LKT was noted in Group I calves following challenge suggesting that no LKT or partial LKT protein was produced by *lkt*⁻ -strain and hence was not

responsible for the results obtained in this study. A slightly elevated LKT titer which was observed in *lkt*$^{-}$ -challenged calves was considered in response to the resident normal population of *M. haemolytica* in the nasopharyngeal region. *Mannheimia haemolytica* is an opportunistic pathogen and shipping fever is a multifacotrial disease in which stress and viral predisposition play important role. In the absence of such predisposing factors, it is difficult to reproduce disease in experimental conditions [6]. Moreover, though intratracheal administration of *M. haemolytica* has been used extensively to produce disease experimentally; it is not a natural method of infection where prior nasopharyngeal colonization is essential for production of natural disease.

In summary, we challenged calves with the wild type and LKT-deficient *M. haemolytica*. Following intratracheal inoculation, inflammatory cells were separated from the BAL collected on day -4, 1, 3, and 6. The effects on inflammatory cell functions were measured by quantifying cytokine response using ELISA and real-time RT-PCR. Though a statistically significant difference was not observed in the expression level of various cytokines between calves challenged with the wt and *lkt*$^{-}$ -strain, wt-strain challenged calves exhibited higher clinical score compared to calves challenged with *lkt*$^{-}$ -strain. The results presented in this study also demonstrate that virulence factors other than LKT, in particular LPS, play an important role in the disease and through the use of multiple virulence factors *M. haemolytica* has a selective effect on the induction of cytokine mRNA synthesis by inflammatory cells.

The study was conducted under animal use protocol #182 from the Oklahoma State University Institutional Animal Care and Use Committee.

References

1. Wikse SE. Feedlot cattle pneumonia. *The Veterinary clinics of North America* 1985;1:289-310.

2. Confer AW, Panciera RJ, Clinkenbeard KD, et al. Molecular aspects of virulence of Pasteurella haemolytica. *Canadian journal of veterinary research = Revue canadienne de recherche veterinaire* 1990;54 Suppl:S48-52.

3. Highlander SK. Molecular genetic analysis of virulence in Mannheimia (pasteurella) haemolytica. *Frontiers in bioscience* 2001;6:D1128-1150.

4. Narayanan SK, Nagaraja TG, Chengappa MM, et al. Leukotoxins of gram-negative bacteria. *Veterinary microbiology* 2002;84:337-356.

5. Hsuan SL, Kannan MS, Jeyaseelan S, et al. Pasteurella haemolytica leukotoxin and endotoxin induced cytokine gene expression in bovine alveolar macrophages requires NF-kappaB activation and calcium elevation. *Microbial pathogenesis* 1999;26:263-273.

6. Maheswaran SK, Weiss DJ, Kannan MS, et al. Effects of Pasteurella haemolytica A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals. *Veterinary immunology and immunopathology* 1992;33:51-68.

7. Clinkenbeard KD, Mosier DA, Confer AW. Effects of Pasteurella haemolytica leukotoxin on isolated bovine neutrophils. *Toxicon* 1989;27:797-804.

8. Atapattu DN, Czuprynski CJ. Mannheimia haemolytica leukotoxin induces apoptosis of bovine lymphoblastoid cells (BL-3) via a caspase-9-dependent mitochondrial pathway. *Infection and immunity* 2005;73:5504-5513.

9. Thumbikat P, Dileepan T, Kannan MS, et al. Characterization of Mannheimia (Pasteurella) haemolytica leukotoxin interaction with bovine alveolar macrophage beta2 integrins. *Veterinary research* 2005;36:771-786.

10. Marcatili A, D'Isanto M, Vitiello M, et al. p53 and c-myc activation by Pasteurella haemolytica leukotoxin is correlated with bovine mononuclear cells apoptosis. *The new microbiologica* 2002;25:195-204.

11. Stevens P, Czuprynski C. Dissociation of cytolysis and monokine release by bovine mononuclear phagocytes incubated with Pasteurella haemolytica partially purified leukotoxin and lipopolysaccharide. *Canadian journal of veterinary research = Revue canadienne de recherche veterinaire* 1995;59:110-117.

12. Lafleur RL, Malazdrewich C, Jeyaseelan S, et al. Lipopolysaccharide enhances cytolysis and inflammatory cytokine induction in bovine alveolar macrophages exposed to Pasteurella (Mannheimia) haemolytica leukotoxin. *Microbial pathogenesis* 2001;30:347-357.

13. Clinkenbeard KD, Mosier DA, Confer AW. Transmembrane pore size and role of cell swelling in cytotoxicity caused by Pasteurella haemolytica leukotoxin. *Infection and immunity* 1989;57:420-425.

14. Highlander SK, Fedorova NDH, 2000 #290}, Dusek DM, et al. Inactivation of Pasteurella (Mannheimia) haemolytica leukotoxin causes partial attenuation of virulence in a calf challenge model. *Infection and immunity* 2000;68:3916-3922.

15. Tatum FM, Briggs RE, Sreevatsan SS, et al. Construction of an isogenic leukotoxin deletion mutant of Pasteurella haemolytica serotype 1: characterization and virulence. *Microbial pathogenesis* 1998;24:37-46.

16. Murphy GL, Whitworth LC. Construction of isogenic mutants of Pasteurella haemolytica by allelic replacement. *Gene* 1994;148:101-105.

17. Confer AW, Panciera RJ, Fulton RW, et al. Effect of vaccination with live or killed Pasteurella haemolytica on resistance to experimental bovine pneumonic pasteurellosis. *American journal of veterinary research* 1985;46:342-347.

18. Confer AW, Clinkenbeard KD, Gatewood DM, et al. Serum antibody responses of cattle vaccinated with partially purified native Pasteurella haemolytica leukotoxin. *Vaccine* 1997;15:1423-1429.

19. Moore KW, de Waal Malefyt R, Coffman RL, et al. Interleukin-10 and the interleukin-10 receptor. *Annual review of immunology* 2001;19:683-765.

20. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. *The American review of respiratory disease* 1990;141:471-501.

21. Malazdrewich C, Ames TR, Abrahamsen MS, et al. Pulmonary expression of tumor necrosis factor alpha, interleukin-1 beta, and interleukin-8 in the acute phase of bovine pneumonic pasteurellosis. *Veterinary pathology* 2001;38:297-310.

22. Schindler H, Lutz MB, Rollinghoff M, et al. The production of IFN-gamma by IL-12/IL-18-activated macrophages requires STAT4 signaling and is inhibited by IL-4. *Journal of immunology (Baltimore, Md* 2001;166:3075-3082.

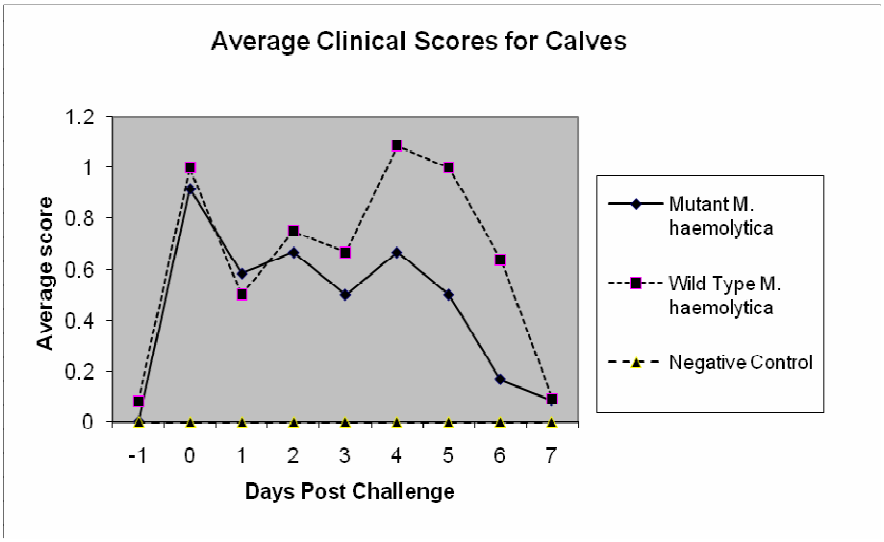

Fig. 1. The mean clinical scores for calves challenged with wt- and *lkt*$^{-}$-strain of *M. haemolytica*. Calves challenged with wt-strain exhibit higher clinical score compared to calves challenged with *lkt*$^{-}$- *strain.*

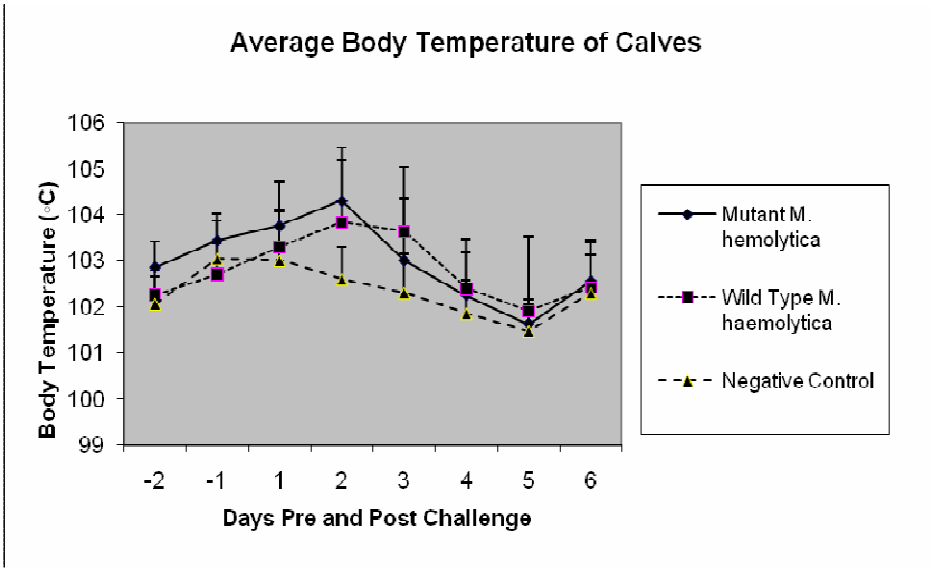

Fig. 2 The mean body temperature of calves challenged with wt- and *lkt* - strain of *M. haemolytica*.

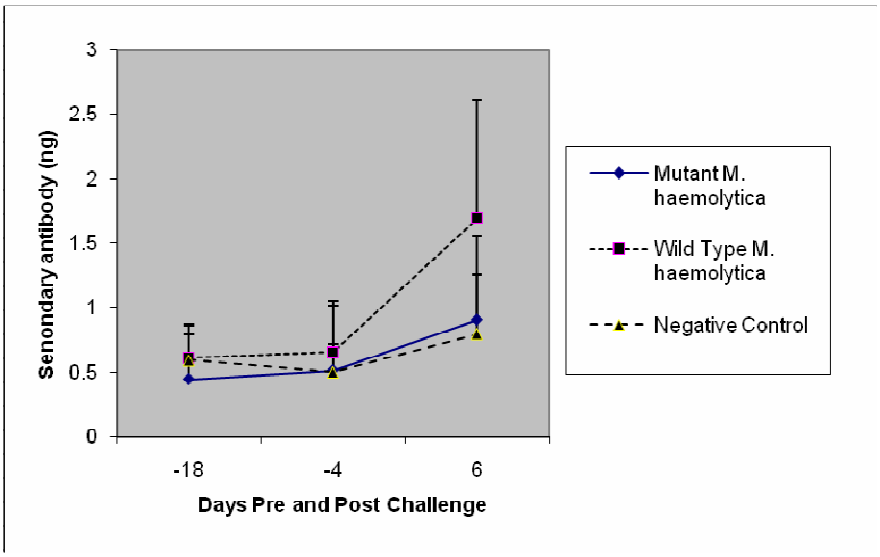

Fig. 3 Results of LKT ELISA on serum obtained from calves pre and post- inoculation.

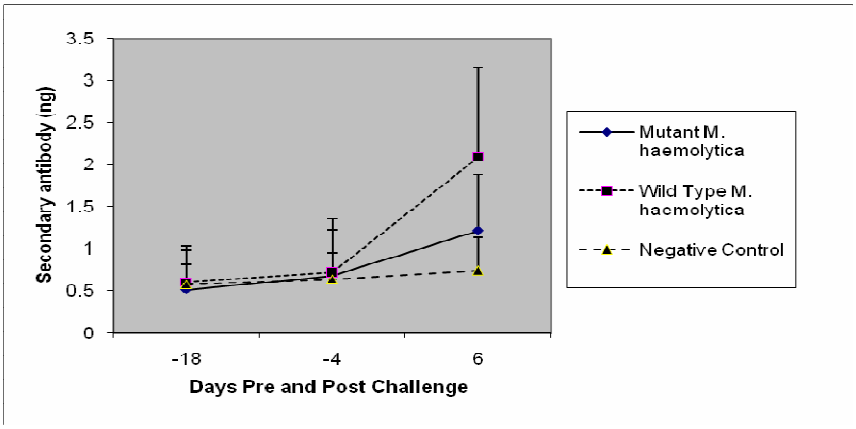

Fig. 4 Results of whole cell (*M. haemolytica*) ELISA on serum obtained from calves pre and post-inoculation.

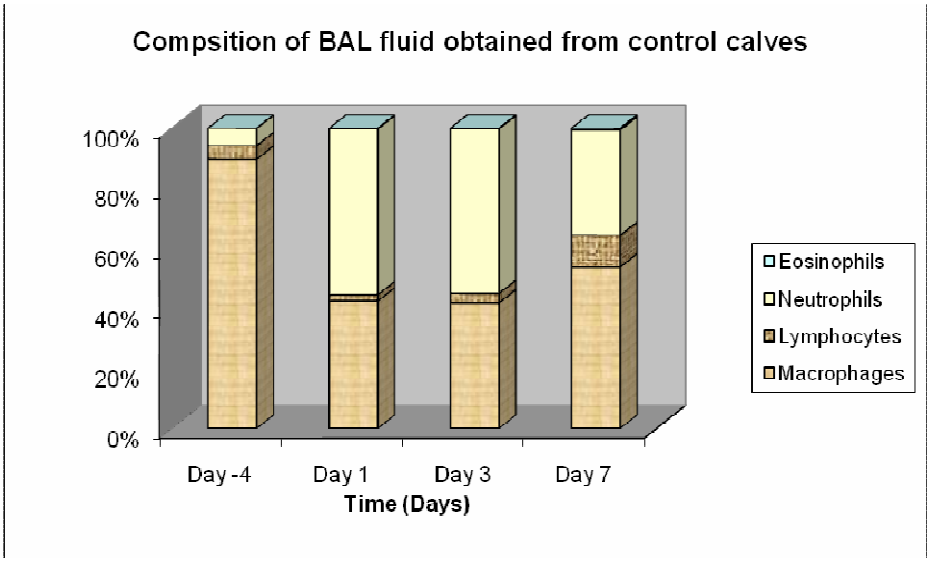

Fig. 5. Composition of BAL fluid obtained from control Group III calves challenged intratracheally with 25 ml of sterile PBS.

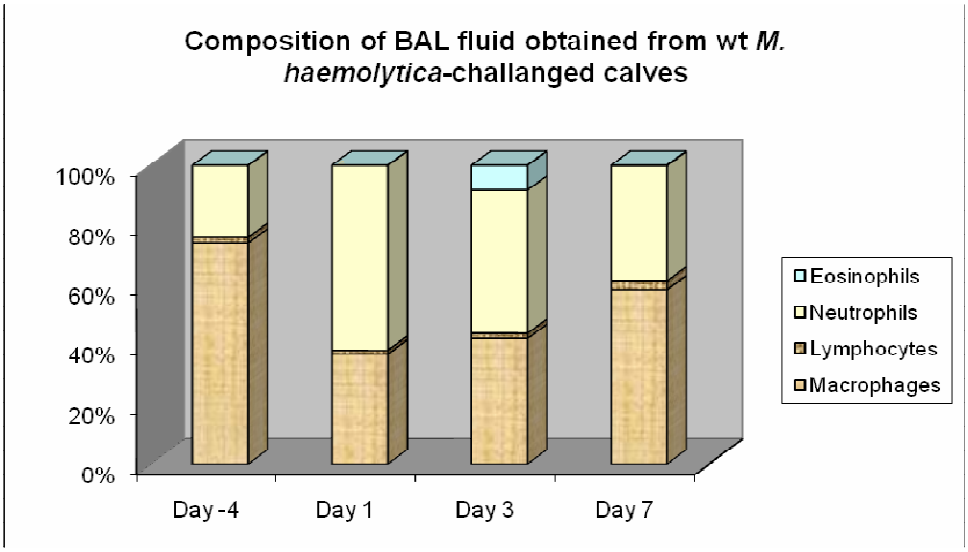

Fig. 6. Composition of BAL fluid obtained from Group II calves challenged intratracheally with 25 ml PBS containing 0.31 x 10^9 cfu/ml of wt *M. haemolytica*.

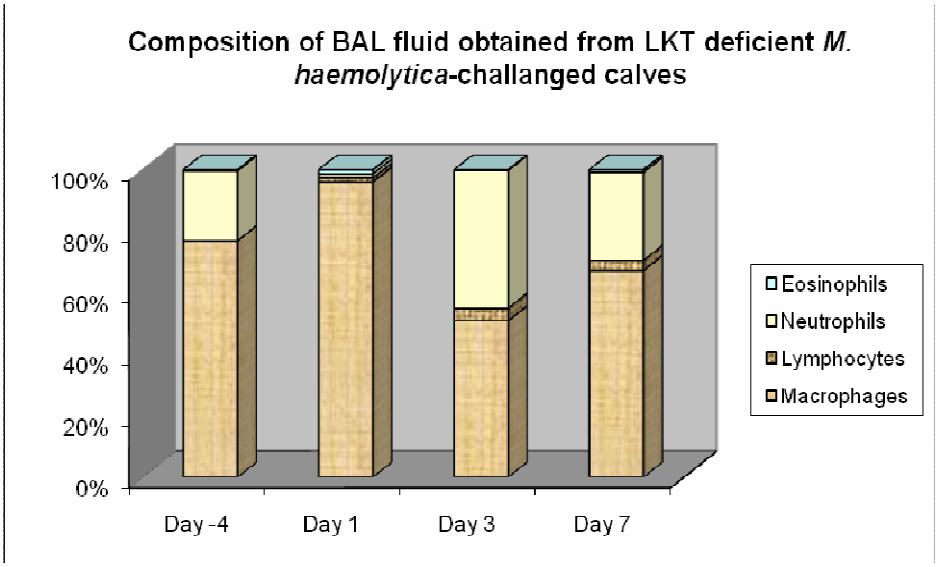

Fig. 7. Composition of BAL fluid obtained from Group I calves challenged intratracheally with 25 ml PBS containing 0.44 x 10^9 cfu/ml of LKT deficient (*lkt*$^-$) *M. haemolytica*.

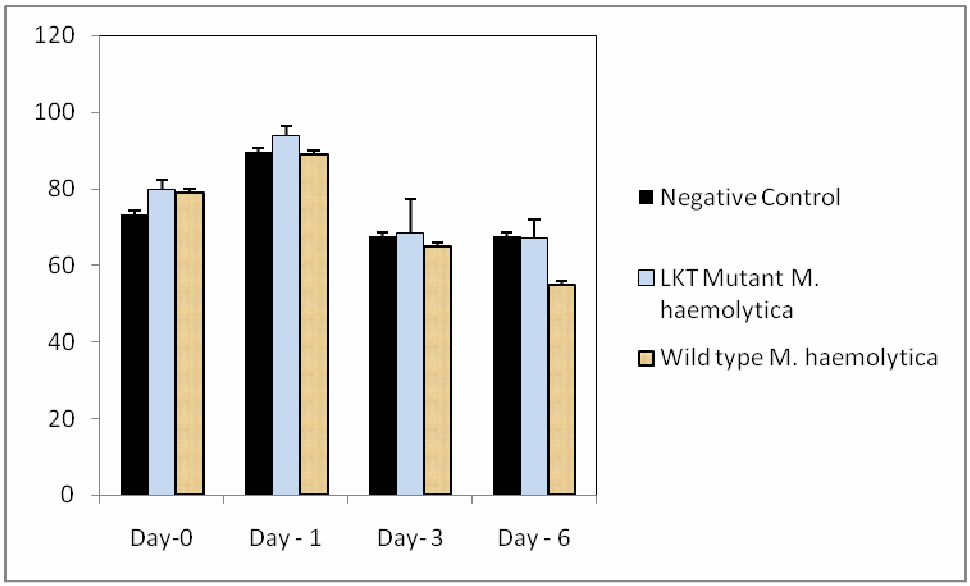

Fig. 8 Quantification of TNF protein in BAL fluid obtained from calves challenged with LKT deficient (*lkt*) and wild type (wt) *M. haemolytica*. The calves were challenged with 25 ml PBS containing 4.4 x 10^8 bacteria/ml and 3.1 x 10^8 bacteria/ml for *lkt*$^-$ and wt, respectively. BAL fluid was harvested at the indicated time points (X-axis) and assayed for the presence of TNF protein (pg/ml) using ELISA. Data are the means ± SEM of samples obtained from 12 different animals per group.

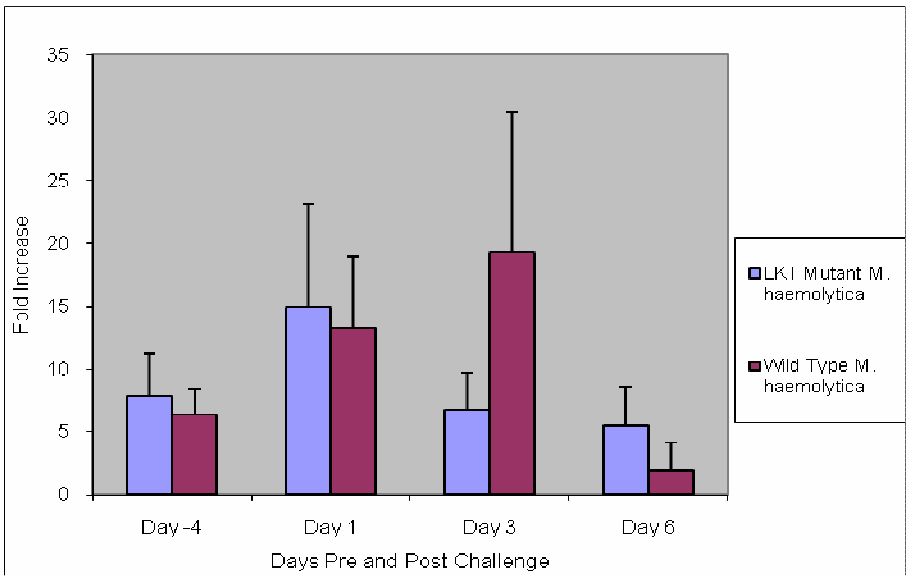

Fig. 9. Fig. Quantification of IL- 8 mRNA production by inflammatory cells obtained from the BAL fluid of calves challenged with LKT deficient (*lkt*$^-$) and wild type (wt) *M. haemolytica*. The calves were challenged with 25 ml PBS containing 0.44 x 10^9 bacteria/ml and 0.31 x 10^9 bacteria/ml for *lkt*$^-$ and wt, respectively. BAL cells were harvested at the indicated time points (X-axis) and assayed for the presence of IL- 8 mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase (Y-axis). Data are the means ± SEM of samples obtained from 12 different animals per group.

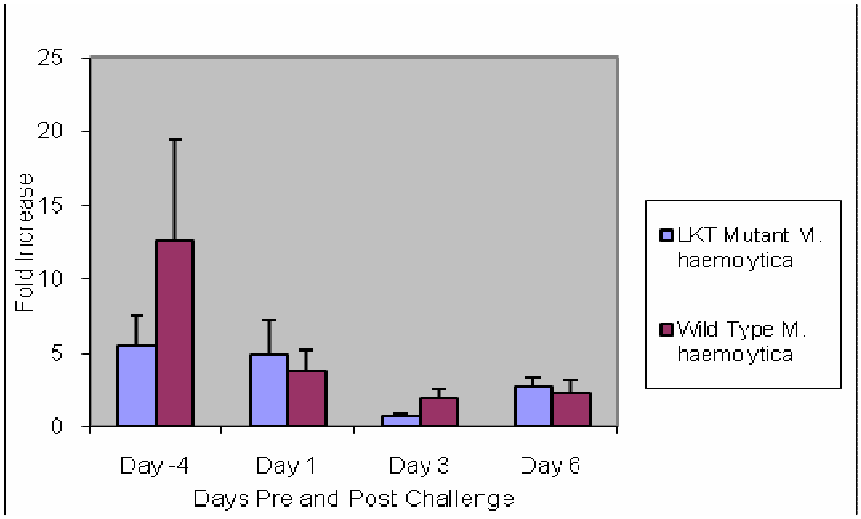

Fig. 10. Quantification of IL- 1 mRNA production by inflammatory cells obtained from the BAL fluid of calves challenged with LKT deficient (*lkt*$^-$) and wild type (wt) *M. haemolytica*. The calves were challenged with 25 ml PBS containing 0.44 x 10^9 bacteria/ml and 0.31 x 10^9 bacteria/ml for *lkt*$^-$ and wt, respectively. BAL cells were harvested at the indicated time points (X-axis) and assayed for the presence of IL- 1 mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase (Y-axis). Data are the means ± SEM of samples obtained from 12 different animals per group.

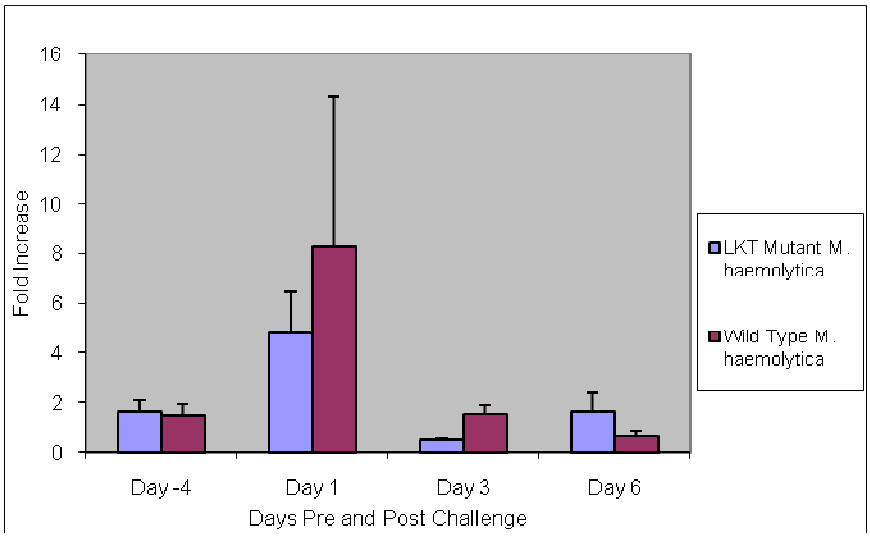

Fig. 11. Quantification of TNFά mRNA production by inflammatory cells obtained from the BAL fluid of calves challenged with LKT deficient (*lkt*$^-$) and wild type (wt) *M. haemolytica*. The calves were challenged with 25 ml PBS containing 0.44 x 10^9 bacteria/ml and 0.31 x 10^9 bacteria/ml for *lkt*$^-$ and wt, respectively. BAL cells were harvested at the indicated time points (X-axis) and assayed for the presence of TNFά mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase (Y-axis). Data are the means ± SEM of samples obtained from 12 different animals per group.

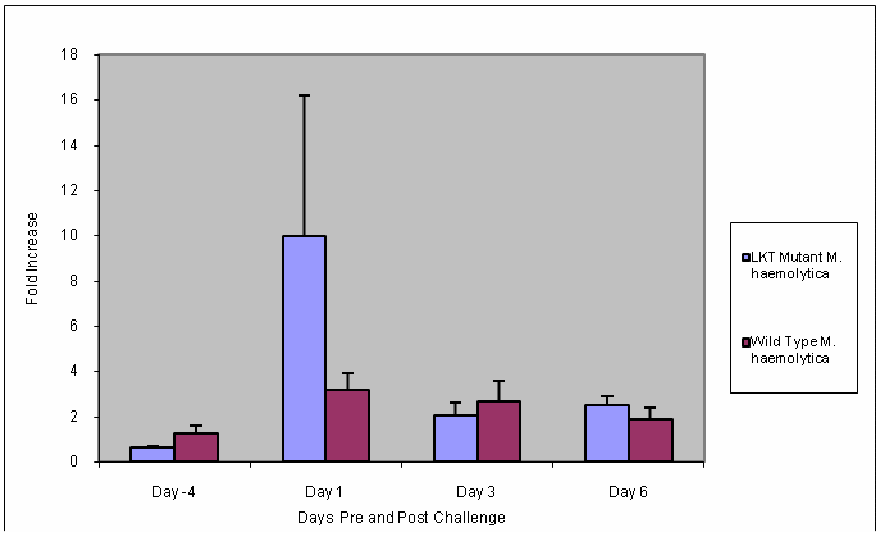

Fig. 12. Quantification of IL- 10 mRNA production by inflammatory cells obtained from the BAL fluid of calves challenged with LKT deficient (*lkt*$^-$) and wild type (wt) *M. haemolytica*. The calves were challenged with 25 ml PBS containing 0.44 x 10^9 bacteria/ml and 0.31 x 10^9 bacteria/ml for *lkt*$^-$ and wt, respectively. BAL cells were harvested at the indicated time points (X-axis) and assayed for the presence of IL- 10 mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase (Y-axis). Data are the means ± SEM of samples obtained from 12 different animals per group.

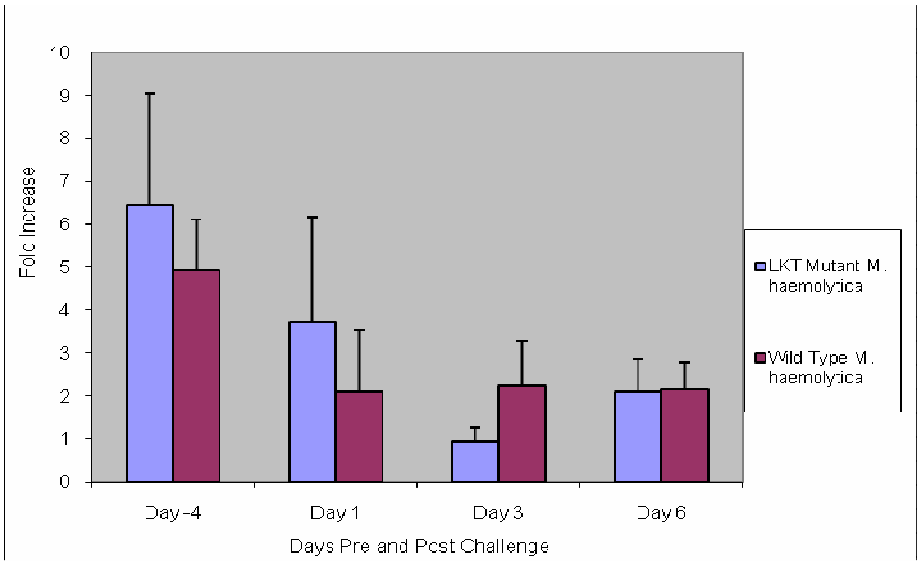

Fig. 13. Quantification of IL- 12 mRNA production by inflammatory cells obtained from the BAL fluid of calves challenged with LKT deficient (*lkt*$^-$) and wild type (wt) *M. haemolytica*. The calves were challenged with 25 ml PBS containing 0.44 x 10^9 bacteria/ml and 0.31 x 10^9 bacteria/ml for *lkt*$^-$ and wt, respectively. BAL cells were harvested at the indicated time points (X-axis) and assayed for the presence of IL- 12 mRNA using RT-PCR. mRNA expression data were normalized to the expression of ß-actin mRNA and the accumulation of cytokine mRNA was expressed as fold increase (Y-axis). Data are the means ± SEM of samples obtained from 12 different animals per group.

References

1. Confer AW, Panciera RJ, Clinkenbeard KD et al. Molecular aspects of virulence of Pasteurella haemolytica. Can J Vet Res 1990;54 Suppl:S48-52.

2. Highlander SK. Molecular genetic analysis of virulence in Mannheimia (pasteurella) haemolytica. Front Biosci 2001;6:D1128-50.

3. Marcatili A, D'Isanto M, Vitiello M et al. p53 and c-myc activation by Pasteurella haemolytica leukotoxin is correlated with bovine mononuclear cells apoptosis. New Microbiol 2002;25(2):195-204.

4. Stevens P, Czuprynski C. Dissociation of cytolysis and monokine release by bovine mononuclear phagocytes incubated with Pasteurella haemolytica partially purified leukotoxin and lipopolysaccharide. Can J Vet Res 1995;59(2):110-7.

5. Lafleur RL, Malazdrewich C, Jeyaseelan S et al. Lipopolysaccharide enhances cytolysis and inflammatory cytokine induction in bovine alveolar macrophages exposed to Pasteurella (Mannheimia) haemolytica leukotoxin. Microb Pathog 2001;30(6):347-57.

6. Highlander SK, Fedorova ND, Dusek DM et al. Inactivation of Pasteurella (Mannheimia) haemolytica leukotoxin causes partial attenuation of virulence in a calf challenge model. Infect Immun 2000;68(7):3916-22.

7. Tatum FM, Briggs RE, Sreevatsan SS et al. Construction of an isogenic leukotoxin deletion mutant of Pasteurella haemolytica serotype 1: characterization and virulence. Microb Pathog 1998;24(1):37-46.

8. Malazdrewich C, Ames TR, Abrahamsen MS et al. Pulmonary expression of tumor necrosis factor alpha, interleukin-1 beta, and interleukin-8 in the acute phase of bovine pneumonic pasteurellosis. Vet Pathol 2001;38(3):297-310.

9. Moore KW, de Waal Malefyt R, Coffman RL et al. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 2001;19:683-765.

10. Murphy GL, Whitworth LC. Construction of isogenic mutants of Pasteurella haemolytica by allelic replacement. Gene 1994;148(1):101-5.

11. Confer AW, Ayalew S, Panciera RJ et al. Recombinant Mannheimia haemolytica serotype 1 outer membrane protein PlpE enhances commercial M. haemolytica vaccine-induced resistance against serotype 6 challenge. Vaccine 2006;24(13):2248-55.

12. Rottman JB, Tompkins WA, Tompkins MB. A reverse transcription-quantitative competitive polymerase chain reaction (RT-qcPCR) technique to measure cytokine gene expression in domestic mammals. Vet Pathol 1996;33(2):242-8.

13. Brown WC, Zhu D, Shkap V et al. The repertoire of Anaplasma marginale antigens recognized by CD4(+) T-lymphocyte clones from protectively immunized cattle is diverse and includes major surface protein 2 (MSP-2) and MSP-3. Infect Immun 1998;66(11):5414-22.

14. Confer AW, Panciera RJ, Fulton RW et al. Effect of vaccination with live or killed Pasteurella haemolytica on resistance to experimental bovine pneumonic pasteurellosis. Am J Vet Res 1985;46(2):342-7.

15. Confer AW, Clinkenbeard KD, Gatewood DM et al. Serum antibody responses of cattle vaccinated with partially purified native Pasteurella haemolytica leukotoxin. Vaccine 1997;15(12-13):1423-9.

16. Maheswaran SK, Weiss DJ, Kannan MS et al. Effects of Pasteurella haemolytica A1 leukotoxin on bovine neutrophils: degranulation and generation of oxygen-derived free radicals. Vet Immunol Immunopathol 1992;33(1-2):51-68.

17. Sibille Y, Reynolds HY. Macrophages and polymorphonuclear neutrophils in lung defense and injury. Am Rev Respir Dis 1990;141(2):471-501.

18. Welch RA, Forestier C, Lobo A et al. The synthesis and function of the Escherichia coli hemolysin and related RTX exotoxins. FEMS Microbiol Immunol 1992;5(1-3):29-36.

19. Moxley RA, Berberov EM, Francis DH et al. Pathogenicity of an enterotoxigenic Escherichia coli hemolysin (hlyA) mutant in gnotobiotic piglets. Infect Immun 1998;66(10):5031-5.

20. Thumbikat P, Briggs RE, Kannan MS et al. Biological effects of two genetically defined leukotoxin mutants of Mannheimia haemolytica. Microb Pathog 2003;34(5):217-26.

21. Singh K, Confer AW, Rizzi T et al. Cytokine mRNA modulation in bovine alveolar macrophages challenged with the wild type and leukotoxin mutant *Mannhemia haemolytica* . 2008.

22. Caswell JL, Middleton DM, Sorden SD et al. Expression of the neutrophil chemoattractant interleukin-8 in the lesions of bovine pneumonic pasteurellosis. Vet Pathol 1998;35(2):124-31.

23. Mitchell GB, Albright BN, Caswell JL. Effect of interleukin-8 and granulocyte colony-stimulating factor on priming and activation of bovine neutrophils. Infect Immun 2003;71(4):1643-9.

24. Nylen S, Sacks D. Interleukin-10 and the pathogenesis of human visceral leishmaniasis. Trends Immunol 2007;28(9):378-84.

25. Lo RY, Sathiamoorthy S, Shewen PE. Analysis of in vivo expressed genes in Mannheimia haemolytica A1. FEMS Microbiol Lett 2006;265(1):18-25.

CHAPTER V

CONCLUSIONS

1. *In vitro* study: we observed an overall increased mRNA expression of cytokines TNFα, IL-1ß, IL-8, IL-10 and IL-12 when bovine alveolar macrophages were challenged with LKT-deficient strain compared to the wt-strain of *M. haemolytica*. LKT-induced cell cytotoxicity was observed in BAM which suggested that the difference in cytokine mRNA expression was due to LKT-induced cell cytotoxicity and cytokine expression is also mediated by LPS and other virulence factors.

2. *In vivo* study: we challenged calves with the wild type and LKT-deficient *M. haemolytica*. Following intratracheal inoculation, inflammatory cells were separated from the bronchioalveolar lavage collected on day -4, 1, 3, and 6. The effects on leukocyte function were measured by quantifying cytokines IL-1β, IL-8, IL-10, IL-12 and TNFα response using ELISA and real-time RT-PCR. Calves challenged with wt-strain had a higher mean clinical score and one death compared to calves challenged with the LKT-deficient strain. The composition of bronchioalveolar lavage fluid was variable between the groups. Though a statistically significant difference in the expression level of cytokines was not observed between the groups challenged with LKT-deficient strain and wt-strain of *M. haemolytica,* increased expression of IL- 8 and IL- 10 was observed in wild type and LKT-deficient *M. haemolytica* challenged calves, respectively. The expression of these cytokines may be correlated with the clinical severity in calves.

3. The results presented in these *in vitro* and *in vivo* studies demonstrate that virulence factors other than LKT, in particular LPS, play an important role in BPP and through the use of multiple virulence factors *M. haemolytica* has a selective effect on the induction of various cytokine mRNA synthesis by inflammatory cells.

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Findings and Conclusions:

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