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Mannheimia haemolytica in bovine respiratory disease: immunogens, potential immunogens, and vaccines

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Review

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Abstract

Mannheimia haemolytica is the major cause of severe pneumonia in bovine respiratory disease (BRD). Early *M. haemolytica* bacterins were either ineffective or even enhanced disease in vaccinated cattle, which led to studies of the bacterium's virulence factors and potential immunogens to determine ways to improve vaccines. Studies have focused on the capsule, lipopolysaccharide, various adhesins, extracellular enzymes, outer membrane proteins, and leukotoxin (LKT) resulting in a strong database for understanding immune responses to the bacterium and production of more efficacious vaccines. The importance of immunity to LKT and to surface antigens in stimulating immunity led to studies of individual native or recombinant antigens, bacterial extracts, live-attenuated or mutant organisms, culture supernatants, combined bacterin-toxoids, outer membrane vesicles, and bacterial ghosts. Efficacy of several of these potential vaccines can be shown following experimental *M. haemolytica* challenge; however, efficacy in field trials is harder to determine due to the complexity of factors and etiologic agents involved in naturally occurring BRD. Studies of potential vaccines have led current commercial vaccines, which are composed primarily of culture supernatant, bacterin-toxoid, or live mutant bacteria. Several of those can be augmented experimentally by addition of recombinant LKT or outer membrane proteins.

Introduction

Mannheimia haemolytica (formerly *Pasteurella haemolytica* Biotype A) is a ruminant pathogen traditionally associated with severe respiratory disease of domestic sheep, bighorn sheep, and cattle, as well as septicemia in lambs and mastitis in ewes (Caswell and Williams, 2007; Dassanayake *et al*., 2009, 2010; Singh *et al*., 2011; Besser *et al*., 2013; Gelasakis *et al*., 2015). Bovine respiratory disease (BRD) is the major cause of beef cattle morbidity, mortality, and reduced production and costing the US cattle industry approximately $1 billion per year, when drugs, labor costs, decreased production, and animal death losses are taken into account (Marshall and Levy, 2011). In healthy cattle, *M. haemolytica* is a natural inhabitant of the upper respiratory tract including the nasal passages, nasopharynx, and tonsils; paranasal sinuses are predominately sterile, and *M. haemolytica* was isolated from transtracheal fluids from 13.1% of healthy cattle (Frank and Briggs, 1992; Frank *et al*., 1995; Murray *et al*., 2017; Timsit *et al*., 2017). *M. haemolytica* live within biofilms on the upper respiratory mucosa (Olson *et al*., 2002; Boukahil and Czuprynski, 2015, 2016, 2018). Multiple surface adhesins, including several surface proteins, fimbriae, and the polysaccharide capsule, are responsible for adherence of *M. haemolytica* to the upper respiratory mucosa and colonization (Morck *et al*., 1988; Jaramillo *et al*., 2000; Lo, 2001; Gioia *et al*., 2006; Daigneault and Lo, 2009; Kisiela and Czuprynski, 2009). Early association of severe respiratory disease in stockyards and shipping earned the disease such names as stockyards pneumonia, shipping fever, and transit fever, whereas the name shipping fever is commonly used today for BRD in stressed beef cattle (Carter, 1967; Mosier *et al*., 1989*a*). Stress caused by environmental changes, shipping, weaning, comingling, and viral infections cause the bacterium to proliferate, to release from biofilms on the upper respiratory surface, and to be inhaled into the lower respiratory tract. Recently, an *in vitro* model demonstrated dispersal of *M. haemolytica* from biofilms treated with stress-related substances, epinephrine and to a lesser extent norepinephrine and substance P (Pillai *et al*., 2018). When host defenses are overcome, the bacterium can precipitate severe fibrinous bronchopneumonia and death (Grey and Thomson, 1971; Frank and Smith, 1983; Frank *et al*., 1987; Caswell and Williams, 2007; Booker *et al*., 2008; Panciera and Confer, 2010; Singh *et al*., 2011). There are several serotypes of *M. haemolytica* (see "The organism", below), and in severe, often fatal, pneumonia in cattle, especially in weaned beef cattle, Serotype 1 (S1) is most commonly isolated from sick cattle or from lesions of pneumonia (Purdy *et al*., 1997*a*; Al-Ghamdi *et al*., 2000; Katsuda *et al*., 2008; Panciera and Confer,

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2010; Klima *et al*., 2014). Serotypes 2 and 6 are common causes of sheep pneumonia (Odendaal and Henton, 1995). Serotype 6 is isolated from BRD cases approximately 20% of the time or less, whereas S2 is often isolated from the nasal passages in high concentration in healthy, non-stressed cattle, but infrequently causes bovine pneumonia (Frank and Smith, 1983).

The organism

M. haemolytica is a Gram-negative, non-motile, non-spore-forming, facultative anaerobic, weakly hemolytic coccobacillus, and a member of the family Pasteurellaceae (Rice *et al*., 2007). Several virulence factors are produced by the bacterium, notably endotoxin and leukotoxin (LKT) (see section below, "Virulence factors and potential immunogens"). It primarily infects ruminant species causing pneumonia, septicemia, or mastitis (Singh *et al*., 2011). Historically, hemolytic strains of bacteria were isolated from bovine pneumonia in the 1920s and studied under the name *Bacillus boviseptica* (Jones, 1921; Jones and Little, 1921). The organism was later named *Pasteurella haemolytica* to distinguish it from non-hemolytic *Pasteurella multocida* (Newsome and Cross, 1932). Studies to distinguish *P. haemolytica* strains serologically led to the classification of the bacterium into 16 serotypes using hemagglutination assays or a rapid direct, plate agglutination test (Carter, 1956; Biberstein *et al*., 1960; Biberstein, 1965, 1978; Frank and Wessman, 1978). Smith divided *P. haemolytica* into biotypes A and T, whereas biotype A strains fermented arabinose, and biotype T strains fermented trehalose (Smith, 1959). In 1995, serotype A17 was added resulting in 13 serotypes as biotype A and 4 as biotype T (Younan and Fodar, 1995).

Bingham *et al*. (1990) noted that biotype A strains were related by DNA homology, and biotype T strains were related; however, biotype A and T strains had a little genetic relationship. Sneath and Stevens (1990) proposed the name *Pasteurella trehalosi* for biotype T strains, and Blackall *et al*. (2007) later demonstrated genotypically and phenotypically that those bacteria were distinct from other *Pasteurella* spp. and transferred *P. trehalosi* to a new genus as *Bibersteinia trehalosi*.

In 1999, the genus *Mannheimia* was proposed, and through DNA–DNA hybridization and 16S rRNA gene sequencing, all but one of the A biotypes were designated *M. haemolytica*. *Pasteurella haemolytica* A11 was unique and assigned a separate species, *Mannheimia glucosida* (Angen *et al*., 1999). Therefore, *M. haemolytica* consists of the previous *P. haemolytica* biotype A Serotypes 1, 2, 5–9, 12–14, 16 and 17 along with untypable strains (Katsuda *et al*., 2008). Although all *M. haemolytica* serotypes are derived from biotype A, the designation of *M. haemolytica* serotypes as A1, A2, etc., often continues, even though it is redundant to include the biotype designation. Therefore, designation as Serotype 1 (S1), Serotype 2 (S2), Serotype 6 (S6), etc. seems appropriate. Serotypes 1 and 6 are closely related genetically and immunologically (Morton *et al*., 1995; Confer *et al*., 2006; Crouch *et al*., 2012; Klima *et al*., 2016). Despite genetic similarities among serotypes, sequencing of specific genes demonstrated diversity among similar serotypes and among bovine and ovine isolates. Davies *et al*. (Davies *et al*., 2001; Davies and Lee, 2004) found that diversity exists in the major outer membrane protein OmpA between bovine and ovine isolates, and LKT diversity exists among ovine strains of *M. haemolytica*. Lawrence *et al*. (2010) demonstrated an overall dissimilarity of 12% among LKT genes from several isolates. Ayalew *et al*. (2006) demonstrated that major outer membrane lipoprotein PlpE was highly conserved among *M. haemolytica* S1 and S6 strains and highly diverse among S2 strains. Comparison of the transferrin-binding protein operons (*tbpBA*) of *M. haemolytica* S1 and S6, *M. glucosida*, and *B. trehalosi* revealed the existence of a common gene pool among the organisms. In addition, *tbpBA* alleles of bovine *M. haemolytica* S1 and S6 are closely related to ovine origin strains (Lee and Davies, 2011). A multiplex polymerase chain reaction (PCR) test for separating Serotypes 1, 2, and 6 was recently reported (Klima *et al*., 2017).

Virulence factors and potential immunogens

M. haemolytica produces virulence factors that promote lung colonization, stimulate the production of inflammatory mediators, enhance evasion of host defense mechanisms, and stimulate an *M. haemolytica* – specific immune response. If the innate immune response fails to curtail pulmonary colonization, stimulation of pro-inflammatory cytokines and bacterial evasion of host defenses can promote development of pneumonia (Rice *et al*., 2007; Srikumaran *et al*., 2007; Singh *et al*., 2011). Virulence factors consist of capsular polysaccharides (CPS), lipopolysaccharide (LPS), adhesins, outer membrane proteins, iron-binding proteins, secreted enzymes, endotoxin, and the ruminant-specific repeats-in-toxin (RTX), LKT (Table 1) (Confer, 2009). Klima *et al*. (2014) used PCR to screen for six *M. haemolytica* virulence genes including LKT (*lktC*), a putative adhesin (*adhs*), outer-membrane lipoprotein Gs60 (*gs60*), O-sialoglycoprotease (*gcp*), transferrin-binding protein B (*tbpB*), and UDP-N-acetyl-D-glucosamine-2-epimerase (*nmaA*). Each gene was identified in all *M. haemolytica* S1 and S6 isolates from both healthy and sick cattle. Finally, the importance of the various virulence factors, including CPS, LPS, adhesins, outer membrane proteins, iron-binding proteins, secreted enzymes, endotoxin, and LKT, in pathogenesis makes them targets of the host immune response and, therefore, potential targets for vaccine development.

Adhesins

Specific *M. haemolytica* adhesins include a 68 kDa glycoprotein, N-acetyl-D-glucosamine, that mediates adherence to tracheal epithelial cells and activates the oxidative burst of bovine neutrophils through a 165-kDa glycoprotein receptor (Jaramillo *et al*., 2000; De la Mora *et al*., 2006; De la Mora *et al*., 2007). Heat-modifiable outer membrane protein A (OmpA) mediates *M. haemolytica* binding to bronchial epithelial cells and binds fibronectin, whereas addition of anti-OmpA antibodies reduces biofilm formation *in vitro* (Lo and Sorensen, 2007; Kisiela and Czuprynski, 2009; Boukahil and Czuprynski, 2015). In addition, the 30-kDa surface Lipoprotein 1 was identified as important for *M. haemolytica* adhesion to the bronchial epithelium (Kisiela and Czuprynski, 2009). *M. haemolytica* capsule has antiphagocytic properties and may function as an adhesin (Morck *et al*., 1988, 1989; Chae *et al*., 1990; Whiteley *et al*., 1990). Several other *M. haemolytica* adhesin proteins have been studied. A collagen-binding autotransporter adhesin was identified, and anti-autotransporter antibodies were in sera from *M. haemolytica*-challenged cattle (Daigneault and Lo, 2009). Large, rigid fimbriae with subunit proteins of approximately 35 kDa and type IV pili were identified, and these structures often serve as adhesins in numerous bacterial species (Potter *et al*., 1988; Morck *et al*., 1989; Lawrence *et al*., 2010). Filamentous hemagglutinins (Fha) are major surface proteins associated with adhesion of various

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Table 1. Immunogens and potential immunogens of *Mannheimia haemolytica*

Antigen	Origin or source	Virulence Factor	Immunogenic	Key Reference
Neuraminidase	Extracellular	Hydrolyzes sialic acid residues on cell surfaces	Moderate	Straus and Purdy (1995)
Sialoglycoprotease	Extracellular	Cleaves cell surface glycoproteins	Strong	Lee *et al.* (1994*b*)
Lipoprotein 1	Membrane	Adhesin	Moderate	Kisiela and Czuprynski (2009)
Filamentous hemagglutinin	Membrane	Adhesin	Strong	Klima *et al.* (2018)
Serotype 1-specific antigen	Outer membrane	Possible adhesin	Strong	Gonzalez-Rayos *et al.* (1986)
OmpA	Outer membrane	Adhesin Binds lactoferrin	Strong	Mahasreshti *et al.* (1997)
PlpE	Outer membrane	Unknown	Strong	Pandher *et al.* (1998)
Transferrin binding proteins A & B	Outer membrane	Remove iron from transferrin	Strong	Deneer and Potter (1989)
PlpF	Outer membrane	Unknown	Strong	Ayalew *et al.* (2011*a*)
OmpD15 (Omp85)	Outer membrane	Unknown	Weak	Ayalew *et al.* (2011*b*)
OmpP2	Outer membrane	Unknown	Weak	Ayalew *et al.* (2011*b*)
Gs60	Outer membrane and extracellular	Unknown	Strong	Moore *et al.* (2011)
Leukotoxin	Secreted	Leukocyte necrosis & apoptosis	Strong	Shewen and Wilkie (1982)
Metalloproteases	Secreted	enzymatic	Unknown	Ramirez Rico *et al.* (2017)
Capsule	Surface	Antiphagocytic Adhesin	Weak	Adlam *et al.* (1984)
Lipopolysaccharide	Surface	Pro-inflammatory compound	Lipid A–Weak Polysaccharide - Moderate	Rimsay *et al.* (1981)
Fimbria	Surface	Adhesin	Strong	Potter *et al.* (1988)
N-acetyl-D-glucosamine	Surface	Adhesion	Unknown	Jaramillo *et al.* (2000)

bacteria, especially *Bordetella* spp. (Scheller and Cotter, 2015). Gioia *et al.* (2006) demonstrated sequence homology among genes that code for *M. haemolytica* FhaB, in three *M. haemolytica* strains (Lawrence *et al.*, 2010). Using *in silico* identification and high throughput screening, *M. haemolytica* Fha was identified as a highly immunoreactive protein when screened with sera against serotypes 1, 2, and 6 (Klima *et al.*, 2018). The Serotype 1- specific (SSA-1) antigen was suggested to function as an adhesin. The gene coding for SSA-1 is expressed *in vivo* during lung infection and present in several *M. haemolytica* serotypes as well as in S1. In addition, the protein is highly immunogenic (Gonzalez *et al.*, 1991, 1995; Lawrence *et al.*, 2010; Ayalew *et al.*, 2011*b*; Sathiamoorthy *et al.*, 2011).

Secreted enzymes

M. haemolytica secretes numerous enzymes into culture supernatants and, therefore, likely the respiratory lumens during infection. Several proteases were recently identified in the culture supernatant of *M. haemolytica* S2, and those were primarily cysteine proteases or metalloproteases (Ramirez Rico *et al.*, 2017). A specific, 100 kDa Zn-dependent metalloprotease was identified in the same study.

Neuraminidase (sialidase) is an extracellular protein associated with numerous bacterial species and hydrolyzes sialic acid residues from host mucosal sialoglycoproteins exposing underlying carbohydrate moieties used for bacterial adhesion (Moncla *et al.*, 1990; Lewis and Lewis, 2012). Therefore, neuraminidase is not a bacterial adhesin itself but may enhance bacterial adhesion through modification of cell surfaces allowing adhesins to interact with the surface. *M. haemolytica* neuraminidase was demonstrated to be a large, approximately 160 kDa, extracellular, heat-labile enzyme produced by various serotypes, primarily during stationary growth phase (Frank and Tabatabai, 1981; Straus and Purdy, 1995; Straus *et al.*, 1998; Highlander, 2001). Neuraminidase is produced *in vivo* in *M. haemolytica*-infected cattle as evidenced by the rise in anti-neuraminidase antibodies during infection (Straus *et al.*, 1998).

O-Sialoglycoprotease (also referred to as O-sialoglycoprotein endopeptidase and glycoprotease) is a 35.2 kDa endopeptidase that hydrolyzes peptide bonds within glycoproteins with a marked specificity for sialylated glycoproteins (Otulakowski *et al.*, 1983; Abdullah *et al.*, 1990, 1992; Lee *et al.*, 1994*a*, 1994*b*; Mellors and Lo, 1995). Its enzyme activity was originally identified in *M. haemolytica* culture supernatants, the protein was demonstrated by proteomic analyses in supernatants, and the enzyme was isolated and activity characterized as a neutral metalloprotease (Otulakowski *et al.*, 1983; Abdullah *et al.*, 1992; Ayalew *et al.*, 2017*b*). Subsequently, Lo *et al.* (1994) cloned and expressed the gene as a fusion protein. The sialoglycoprotease gene and glycoprotease activity were associated with numerous *M. haemolytica* serotypes, and homologs of the protein were detected in several

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Gram-negative bacteria; however, secretion in the form of O-sialoglycoprotease was restricted to *M. haemolytica* serotypes (Lee *et al.*, 1994*a*; Lawrence *et al.*, 2010; Klima *et al.*, 2014). Vaccination of calves with recombinant sialoglycoprotease-fusion protein stimulated antibodies against the protein (Shewen *et al.*, 2003). Antibodies to sialoglycoprotease were identified in sera of cattle challenged with live *M. haemolytica* (Lee *et al.*, 1994*b*). The role of sialoglycoprotease in respiratory pathogenesis is yet unknown. It was shown to cleave cell surface glycoproteins CD34 (found on hematopoietic progenitors and endothelium), CD43 (leukosialin a surface protein on leukocytes), CD44 (a hyaluronic receptor serving as a cell adhesin molecule), CD45 (the leukocyte common antigen associated with signal transduction) and platelet selectin (Sutherland *et al.*, 1992*a*, 1992*b*; Norgard *et al.*, 1993; Mellors and Lo, 1995). Lawrence *et al.* (2010) suggested that the enzyme might assist in colonization of the upper respiratory tract.

Proteases that cleave host immunoglobulins have been described in various bacteria, are often produced as a component of autotransporter proteins, and can be secreted into the surrounding milieu (Mistry and Stockley, 2006). Lee and Shewen (1996) demonstrated bovine IgG1 protease activity in *M. haemolytica* culture supernatants that hydrolyzed bovine IgG1 into 39, 12, and 7 kDa bands, had no effect on IgG2, and was inhibited by EDTA, indicating it was a metalloprotease. The authors suggested that sialoglycoprotease might be involved in this process; however, further study on that point has not been documented in the literature. Proteases that cleave IgG have been identified in *Streptococcus pyogenes*, *Pseudomonas aeruginosa, Proteus mirabilis,* and *Staphylococcus aureus* (Brezski and Jordan, 2010; Rungelrath *et al.*, 2017; Wang *et al.*, 2017). A search of the genome databases of 10 *M. haemolytica* failed to demonstrate a putative IgG protease in *M. haemolytica*; however, numerous endopeptidases are present that could potentially cleave IgG (Ayalew, unpublished data, 2017). In addition, Ayalew *et al.* (2017*b*) demonstrated by proteomic analyses a putative IgA protease in *M. haemolytica* culture supernatant. IgA proteases in other bacteria are components of autotransporter molecules, enhance bacterial invasion of mucosal surfaces, and assist in bacterial escape from host defenses (Mistry and Stockley, 2006). In addition, bacterial IgA proteases are immunogenic and stimulate local and systemic antibodies in infected hosts (Morelli *et al.*, 1994; Kirkeby *et al.*, 2000; Kotelnikova *et al.*, 2016).

Leukotoxin

M. haemolytica LKT (originally called cytotoxin) has been the subject of much research since *M. haemolytica* – induced cytotoxic damage of bovine macrophages and neutrophils was described *in vitro,* and the toxin was identified (Benson *et al.*, 1978; Baluyut *et al.*, 1981; Berggren *et al.*, 1981; Shewen and Wilkie, 1982). The LKT is encoded by four genes in the toxin operon, *lktC, lktA, lktB,* and *lktD* (Lo *et al.*, 1987). *lktA* codes for the structural toxin, and *lktC* is involved in activation, whereas products of *lktB* and *lktD* are associated with secretion (Highlander, 2001; Rice *et al.*, 2007). For a detailed description of *M. haemolytica* LKT and its genetics and activities, the reader is referred to one of several review articles (Highlander, 2001; Jeyaseelan *et al.*, 2002; Zecchinon *et al.*, 2005; Rice *et al.*, 2007; Czuprynski, 2009; Singh *et al.*, 2011). Recently, LKT acyl transferase gene (*artJ-lktC*) was used in a multiplex PCR assay that identifies *M. haemolytica*, *P. multocida,* and *Trueperella pyogenes* in infected lungs (Zhang *et al.*, 2017).

After the discovery of *M. haemolytica* LKT, it was characterized as a 104 kDa protein and a member of the RTX family of toxins, which includes the *Escherichia coli* α-hemolysin and numerous toxins from other Gram-negative bacteria (Frey, 2011). The toxic component of LKT resides in the N-terminus, whereas the region stimulating LKT–neutralizing antibodies is localized to a 32 amino acid region near the C-terminus (Lainson *et al.*, 1996; Welch, 2001). The toxin is specific for leukocytes from various ruminant species and not for other cell types or leukocytes from nonruminant animal species; therefore, the name was changed from cytotoxin to LKT (Confer *et al.*, 1990; Jeyaseelan *et al.*, 2002; Narayanan *et al.*, 2002). LKT is secreted into culture supernatants during logarithmic growth phase, binds to the LKT receptor β2 integrins, CD18, and induces dose-related changes in bovine leukocytes (Shewen and Wilkie, 1985; Ambagala *et al.*, 1999; Li *et al.*, 1999; Odendaal and Ellis, 1999; Dassanayake *et al.*, 2007; Tucci *et al.*, 2016). At high LKT concentrations, leukocytes undergo rapid osmotic swelling, membrane pore formation, and necrosis (Clinkenbeard *et al.*, 1989). At reduced doses, LKT can induce leukocyte apoptosis, activate leukocytes with release of proinflammatory cytokines and oxygen-derived free radicals, reduce mitogen-mediated lymphogenesis, and stimulate histamine release from mast cells (Majury and Shewen, 1991; Maheswaran *et al.*, 1992; Adusu *et al.*, 1994; Cudd *et al.*, 2001, 2003; Rice *et al.*, 2007; Singh *et al.*, 2011). Localization of LKT by immunohistochemistry in the lung after challenge demonstrated LKT associated with necrotic leukocytes and cell debris within alveoli (Whiteley *et al.*, 1990). Challenge of cattle with *M. haemolytica* LKT deletion mutants resulted in less severe lesions than with parent strains (Tatum *et al.*, 1998; Highlander *et al.*, 2000). Therefore, due to its many pathologic effects on leukocytes, LKT is considered the most important virulence factor in *M. haemolytica* – induced pneumonia.

M. haemolytica LKT is responsible for hemolysis *in vitro* and is produced by all serotypes of the bacterium with the exception of the occasional LKT – deficient mutant strains that have been described (Shewen and Wilkie, 1983*b*; Murphy *et al.*, 1995; Ayalew *et al.*, 2017*a*). LKT exposure stimulates neutralizing antibodies against the C-terminus region of the molecule. Despite some genetic diversity among LKT molecules, LKT-neutralizing antibodies against one *M. haemolytica* serotype or isolate usually neutralize LKT from another *M. haemolytica* serotype or isolate or from *B. trehalosi* (Gentry *et al.*, 1985; Lainson *et al.*, 1996; Hodgins and Shewen, 1998; Davies and Baillie, 2003; Shewen and Wilkie, 1983*a*, 1983*b*). Recently, however, it was shown that well-characterized LKT neutralizing monoclonal antibody MM601 did not neutralize serotype 2 or *B. trehalosi* serotype 10 LKT, and likewise, the monoclonal antibody raised against *B. trehalosi* T10 did not neutralize serotype 1 and 2 LKT, suggesting differences in their epitopes (Murugananthan *et al.*, 2018). LKT-neutralizing antibodies strongly correlated with resistance against experimental challenge (Gentry *et al.*, 1985; Rice *et al.*, 2007).

Capsule and lipopolysaccharide

M. haemolytica serotypes produce serotype-specific CPS that are on the surface of the bacteria, particularly during logarithmic growth (Corstvet *et al.*, 1982; Rice *et al.*, 2007). S1 CPS is a complex mannose-rich polymer made of a disaccharide repeat of N-acetylmannosaminuronic acid β1,4 linked with N-acetylmannosamine, is moderately immunogenic, and partially protects the bacterium from phagocytosis (Adlam *et al.*, 1984;

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Czuprynski *et al*., 1989, 1991*a*; Chae *et al*., 1990; Conlon and Shewen, 1993; Tigges and Loan, 1993). Purified *M. haemolytica* S1 CPS did not stimulate the release of pro-inflammatory cytokines from monocytes and macrophages (Czuprynski *et al*., 1991*b*). Deposition of CPS into the lungs of sheep resulted in edema and a mild neutrophilic infiltrate with CPS binding to surfactant, whereas in lungs from cattle experimentally challenged with live *M. haemolytica,* CPS localized in alveolar lumens and macrophages, but not within the alveolar wall (Brogden *et al*., 1989; Whiteley *et al*., 1990). Vaccination of calves with purified CPS, with or without other *M. haemolytica* antigens, stimulated significant anti-CPS IgM and IgG antibodies in sera; however, 36% of calves experimentally vaccinated with CPS developed anaphylaxis, and after challenge, lung lesion scores did not significantly correlate with anti-CPS titers (Conlon and Shewen, 1993). IgG1 and IgG2 anti-CPS antibodies were highest in calves vaccinated with CPS in oil adjuvant, whereas IgM anti-CPS antibodies were highest in calves vaccinated with CPS with aluminum hydroxide adjuvant (Tigges and Loan, 1993). In another study, antibodies to purified CPS and to a partially purified saline extract that contained capsule, LPS, and proteins were examined in sera from cattle vaccinated with live or killed *M. haemolytica* (Confer *et al*., 1989). Correlations between high antibodies to CPS and low lesion scores were inconsistent among experiments, whereas important antibodies in the saline extract were likely proteins (Confer *et al*., 1989; Srinand *et al*., 1996*b*).

M. haemolytica LPS has a classical endotoxic activity that stimulates pro-inflammatory mediator production and inflammation. Those result in modification and damage of endothelium, causing vascular leakage, enhancement and depression of leukocyte functions, and complexing with LKT, increasing LKT-receptor production and augmenting LKT activity (Rimsay *et al*., 1981; Confer and Simons, 1986; Paulsen *et al*., 1989; 1990, 1995; Kumar *et al*., 1991; Saban *et al*., 1997; Cutlip *et al*., 1998; Hsuan *et al*., 1999; Li and Clinkenbeard, 1999; Lafleur *et al*., 2001; Leite *et al*., 2003; McClenahan *et al*., 2008). Intratracheal inoculation of *M. haemolytica* resulted in LPS within the cytoplasm of neutrophils, alveolar macrophages, endothelial cells, and pulmonary intravascular macrophages as well as on epithelial cell surfaces, as determined by immunohistochemistry (Whiteley *et al*., 1990). Therefore, LPS is an important virulence factor widely distributed throughout the *M. haemolytica*-infected lung. Using monoclonal antibodies, antigenic similarities were demonstrated in a carbohydrate moiety of LPS extracted from serotypes 1, 5, 6, 7, 8, and 12 (Durham *et al*., 1988). Nuclear magnetic resonance spectroscopy revealed the O-chain polysaccharides of S1, S6, and S9 to be identical and the core oligosaccharides of S1, S6, S8, S9, and S12 are similar (Lacroix *et al*., 1993). Davies *et al.* demonstrated distinct serological differences among LPS molecules extracted from different serotypes of *M. haemolytica* (Davies and Donachie, 1996; Davies *et al*., 1997); however, *M. haemolytica* LPS is poorly immunogenic, and no correlation existed between anti-LPS antibodies and resistance against experimental challenge (Confer *et al*., 1986*b*). This may be because bovine *M. haemolytica* isolates often do not elaborate an O-antigen, which is the most immunogenic component of LPS (Ali *et al*., 1992). Alternatively, the core lipopolysaccharide of *M. haemolytica* LPS has been analyzed, and glycoconjugates of LPS core were shown to be immunogenic in rabbits and stimulated complement-mediated killing of *M. haemolytica* (St Michael *et al*., 2011*a*, 2011*b*).

Outer membrane proteins (OMPs)

The outer membrane of Gram-negative bacteria is a complex structure that assists bacteria to adapt to environmental changes, regulate influx and efflux of nutrients, and coordinate signal transduction (Khalid *et al*., 2008). *M. haemolytica* has a host of proteins in the outer membrane, and many OMPs share partial sequence homology with OMPs from other Gram-negative pathogens (Squire *et al*., 1984; Confer, 1993; Davies *et al*., 1994; Ayalew *et al*., 2010). Squire *et al.* (1984) extracted outer and inner *M. haemolytica* membranes using detergents and identified two major OMPs that were 30 and 42 kDa. Using radioiodination, Morton *et al.* (1996) demonstrated eight surface-exposed *M. haemolytica* proteins. Pandher *et al.* (1999) identified 21 surface-exposed, immunogenic *M. haemolytica* OMPs using Western immunoblots on protease-treated and untreated bacteria. Ayalew *et al.* (2010) demonstrated 55 potentially immunogenic *M. haemolytica* OMPs by immunoproteomic analyses with those proteins potentially involved in cell structure, transport mechanisms, general metabolism, translation or other unknown functions. *M. haemolytica* outer membrane is adaptable with the number and character of the OMP profile changing depending on growth conditions and media (Gatewood *et al*., 1994). OMPs are not virulence factors *per se* with the exception of OmpA and SSA-1, which have adhesin properties, and transferrin binding proteins that procure iron from host transferrin (Potter *et al*., 1999; Kisiela and Czuprynski, 2009; Lawrence *et al*., 2010). The importance of OMPs for this review is as potential immune targets for the production of opsonizing antibodies to *M. haemolytica*. Shewen and Wilkie (1988) demonstrated that vaccine immunity to *M. haemolytica* required both LKT-neutralizing antibodies and opsonizing antibodies to surface antigens. Because antibody responses to CPS and LPS do not appear to correlate with protection against *M. haemolytica* challenge, surface proteins are the more likely targets for stimulating the production of opsonizing antibodies. Several OMPs were examined as potential targets for the development of opsonizing antibodies. In fact, correlations between antibodies against several different OMPs and resistance to experimental challenge have been documented; therefore, opsonizing antibodies directed against multiple OMPs are likely involved in immunity to *M. haemolytica* (Mosier *et al*., 1989*b*).

M. haemolytica OMPs that have been studied to some degree with respect to immunity include OmpA, SSA-1, Gs60, PlpE, TBPs, PlpF, OmpD15, and OmpP2. Three approaches have been taken: (1) determination if antibodies to a specific OMP are present in higher concentration in sera from cattle that recovered from BRD compared with sera from cattle that were never ill, (2) determination if high antibodies to a specific OMP in sera from *M. haemolytica*–vaccinated cattle correlated with low lung lesion scores after experimental challenge, and (3) immunization of cattle with purified or recombinant OMPs either by themselves or in conjunction with a *M. haemolytica* vaccine followed by experimental challenge.

One of the first characterized *M. haemolytica* OMPs was the approximately 104 kDa SSA-1 (Gonzalez-Rayos *et al*., 1986). The protein was identified in *E. coli* expressing plasmids from a *M. haemolytica* gene library using antibodies against *M. haemolytica* culture supernatant. Initial studies indicated that the protein was reasonably specific for *M. haemolytica* S1; however, later studies identified *ssa1* was distributed among seven *M. haemolytica* serotypes, including S1, S2, and S6 (Gonzalez *et al*., 1991). Subsequent studies indicated that the genes encoding SSA-1

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derived from *M. haemolytica* S1 or S2 were identical (Gonzalez *et al.*, 1995). Localization of SSA-1 in the outer membrane was confirmed in studies of *M. haemolytica* outer membrane vesicles (Ayalew *et al.*, 2013; Roier *et al.*, 2013). Recently, Kumar *et al.* (2015) used *ssa1* gene as part of a multiplex PCR for rapid detection of *M. haemolytica* from sheep lungs. We vaccinated mice and cattle with recombinant SSA-1 and demonstrated that it was highly immunogenic resulting in significant increases in antibodies by day 28 after vaccination (Ayalew *et al.*, 2011*b*). Vaccination of mice with recombinant SSA-1 and LKT-PlpE chimeric protein (SAC89) resulted in increased antibody responses to the SSA-1 and to the chimeric protein. Klima *et al.* (2018) demonstrated that using *in silico* identification and high throughput screening of antigenic proteins, SSA-1 was the most immunoreactive of the proteins screened with antisera to *M. haemolytica* serotypes 1, 2, and 6. Challenge of cattle vaccinated with SSA-1 has not been done to our knowledge.

The OmpA Family of outer membrane proteins is a group of genetically related, heat-modifiable, surface-exposed, porin proteins ranging from 30 to 35 kDa that is in the outer membrane of numerous Gram-negative bacteria (Confer and Ayalew, 2013). Members of the OmpA family of proteins are potential vaccine candidates for several bacteria (Pore and Chakrabarti, 2013; Dubey *et al.*, 2016; Zhang *et al.*, 2016). OmpA for many bacteria is among the most numerous OMPs in the outer membrane (Khalid *et al.*, 2008). *M. haemolytica* OmpA is an approximately 30 kDa, heat-modifiable, surface-exposed, highly immunogenic protein with porin activity, and as a member of the OmpA family of proteins, it shares partial homology with heat-modifiable OMPs from numerous bacteria (Khalid *et al.*, 2008; Ayalew *et al.*, 2011*b*; Confer and Ayalew, 2013). As described above, the protein has adhesin properties and was recently identified as binding lactoferrin, therefore, *M. haemolytica* OmpA may assist removal of iron from lactoferrin (Kisiela and Czuprynski, 2009; Samaniego-Barron *et al.*, 2016). *M. haemolytica* OmpA, originally called PomA, was purified and partially characterized, and its gene was cloned, expressed, and sequenced (Mahasreshti *et al.*, 1997; Zeng *et al.*, 1999). Vaccination of cattle with live *M. haemolytica* resulted in high antibodies against *M. haemolytica* OmpA, and adsorption studies demonstrated surface-exposed epitopes (Mahasreshti *et al.*, 1997). Surface exposure of OmpA was corroborated, and two different OmpA subclasses (OmpA1 and OmpA2) with epitopic differences were identified in bovine and ovine isolates, respectively (Hounsome *et al.*, 2011). Vaccination of cattle with recombinant *M. haemolytica* OmpA stimulated high antibody responses with the complement-mediated killing of the bacterium (Ayalew *et al.*, 2011*b*).

Gs60 is a surface-exposed, 60 kDa outer membrane lipoprotein found in *M. haemolytica* culture supernatant (Moore *et al.*, 2011; Ayalew *et al.*, 2017*b*). A fragment of the gene was cloned, characterized, and expressed, and antibodies directed against the expressed protein fragment correlated with resistance against experimental challenge (Weldon *et al.*, 1994). Subsequently, the entire gene was cloned, sequenced, and found in *P. haemolytica* A biotypes (now *M. haemolytica*) and not in T biotypes (now *B. trehalosi*) (Lo and Mellors, 1996). *In vivo* expression of the *gs60* and *lkt* genes were demonstrated within pneumonic lungs of cattle experimentally challenged with *M. haemolytica* (Lo *et al.*, 2006). *Lkt* gene expression increased between 6 and 12 h after challenge, whereas *gs60 gene* expression decreased (Sathiamoorthy *et al.*, 2012). The Gs60 protein was identified as a component of the putative *M. haemolytica* secretome but was not identified in an outer membrane immunoproteomics study of one *M. haemolytica* strain (Lo and Mellors, 1996; Ayalew *et al.*, 2010, 2017*b*). Recombinant Gs60 as a potential vaccine component was studied by several approaches including expression in alfalfa as a potential component of an edible vaccine and incorporation of *M. haemolytica* culture supernatant into immunostimulatory complexes (ISCOMs) with recombinant bovine C3d (Lee *et al.*, 2008; Moore *et al.*, 2011). Feeding of dried Gs60-transgenic alfalfa to rabbits resulted in seroconversion to Gs60 (Lee *et al.*, 2008). *In vivo* cattle studies have not been reported with the Gs60 transgenic alfalfa, and Gs60 was not demonstrated in the ISCOMs. Further, *in vivo* studies demonstrated anti-Gs60 antibodies in sera from calves vaccinated with *M. haemolytica* supernatant vaccines and from calves vaccinated with recombinant Gs60 and challenged (Orouji *et al.*, 2012). In those studies, there were strong correlations between the production of antibodies to LKT and to Gs60, whereas increased antibodies to Gs60 were beneficial in resistance against challenge when anti-LKT antibodies were low.

Several immunogenic, surface-exposed *M. haemolytica* OMPs in the 40–50 kDa range were identified originally by several techniques (Mosier *et al.*, 1989*b*; Morton *et al.*, 1996; Pandher *et al.*, 1999). Of those, the 45-kDa surface-exposed, outer membrane lipoprotein PlpE was extensively studied in our and other laboratories. The *plpE* gene was isolated from a gene library, cloned, sequenced, and expressed (Pandher *et al.*, 1998). Adsorption of serum antibodies with recombinant PlpE reduced the serum-mediated complement-mediated killing of *M. haemolytica*. The protein was named PlpE, because it was the fifth lipoprotein associated with the outer membrane of then *P. haemolytica,* with PlpA-C being three contiguous 28–30 kDa lipoproteins and PlpD being a 31-kDa lipoprotein (Cooney and Lo, 1993; Murphy and Whitworth, 1993; Dabo *et al.*, 1994; Murphy *et al.*, 1998; Nardini *et al.*, 1998). PlpE was identified by immunoproteomics of the outer membrane and is in culture supernatants of *M. haemolytica* propagated under various growth conditions (Ayalew *et al.*, 2010, 2017*b*). Vaccination of cattle with recombinant PlpE plus adjuvant resulted in a reduction in lesion scores of >40% compared with controls after *Mannheimia haemolytica* S1 or S6 challenge (Confer *et al.*, 2003, 2006). In those studies, when incorporated with commercial *M. haemolytica* vaccines, recombinant PlpE enhanced resistance against challenge above that of the commercial vaccine alone. The immunodominant and potentially protective epitopes in PlpE are in a region of eight imperfect repeats of a hexapeptide in the N-terminal region (Ayalew *et al.*, 2004). Antibodies against that region stimulated complement-mediated killing of *M. haemolytica*. The 8 hexapeptide repeats were identical between *M. haemolytica* S1 and S6 isolates, whereas in S2 isolates, the repeats ranged from 3 to 28 hexapeptides (Ayalew *et al.*, 2006). Vaccination of mice and cattle with chimeric proteins composed of the hexapeptide-repeats epitope of PlpE and the neutralizing epitope of LKT stimulated antibodies that bound the surface of *M. haemolytica* and neutralized LKT (Ayalew *et al.*, 2008; Batra *et al.*, 2016*a*). Cattle vaccinated with chimeric protein plus formalin-killed bacteria were highly resistant against experimental challenge (Ayalew *et al.*, 2009; Confer *et al.*, 2009*a*, 2009*b*; Guzman-Brambila *et al.*, 2012).

Iron is essential for bacterial growth and production of virulence factors, and bacteria have acquired several strategies for iron uptake (Gentry *et al.*, 1986; Sheldon *et al.*, 2016). Strategies include extraction of iron from hemoglobin and acquisition

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from transferrin. In addition, through secretion and uptake of siderophores, free iron is obtained. *M. haemolytica* does not produce siderophores; therefore, *M. haemolytica* iron acquisition must be from heme, transferrin, and/or lactoferrin. During low iron concentrations, *M. haemolytica* produces iron-regulated OMPs (IROMPs). Thus, OMP profiles are different among bacteria grown *in vitro* in growth media that is iron-sufficient, iron-deficient, or iron-sufficient with an iron chelator. In addition, growth of bacteria *in vivo,* which is an iron-deficient environment, produces an OMP profile similar to that of the bacterium grown *in vitro* under iron-deficient conditions (Deneer and Potter, 1989; Morck *et al*., 1991; Confer *et al*., 1992, 1995; Davies *et al*., 1994; Gatewood *et al*., 1994). LKT causes hemolysis of bovine erythrocytes, and *in vivo* transcription of two potential hemoglobin receptors, hmbR1 and hmbR2 were demonstrated in *M. haemolytica* within the lung (Murphy *et al*., 1995; Roehrig *et al*., 2007). Iron acquisition from transferrin is a major mechanism used by *M. haemolytica,* and three IROMPs (approximately 70, 77, and 105 kDa) involved in transferrin binding were identified in bacteria grown *in vitro* under iron-restricted conditions or *in vivo* within an intraperitoneal implanted chamber (Deneer and Potter, 1989; Ogunnariwo and Schryvers, 1990; Morck *et al*., 1991; Yu *et al*., 1992; Geschwend *et al*., 1997; Ogunnariwo *et al*., 1997). Western blots using convalescent sera from *M. haemolytica*-infected calves demonstrated antibodies against the three proteins (Deneer and Potter, 1989; Puchalski *et al*., 2013). We demonstrated antibody responses to the 70 and 77 kDa proteins to be significantly higher in live *M. haemolytica*-vaccinated calves than in control calves; however, there was no significant correlation between antibody responses to those proteins and lesion scores following challenge (Confer *et al*., 1995). In another study, calves were vaccinated with either or both native 105 and/or recombinant 70 kDa proteins, termed transferrin-binding proteins (Tbp) A and B, respectively, and challenged (Potter *et al*., 1999). Both proteins were immunogenic and the best protection was in calves vaccinated with both TbpA and TbpB.

Through immunoproteomic analyses using 2D-electrophoresis and Western blots of *M. haemolytica* of outer membrane preparations probed with convalescent cattle sera, we identified several additional OMPs that were of interest for further study (Ayalew *et al*., 2010). These are PlpF, OmpD15, and OmpP2. PlpF is a 29.7 kDa lipoprotein that was identified in the first published *M. haemolytica* sequence as a conserved hypothetical protein (GI 7227128) (Highlander, 2001). The N terminus of PlpF contains a variable number of perfect and imperfect repeats, which varied among S1, S2 and S6 strains, and antigenicity plots predicted those repeats to be highly antigenic (Ayalew *et al*., 2011*a*). The C-terminus half of PlpF shares substantial similarity with a surface-exposed, highly antigenic lipoprotein of *Neisseria meningitidis* (Madico *et al*., 2006). Recombinant PlpF was immunogenic in mice and calves, and anti-PlpF antibodies are associated with complement-mediated killing (Ayalew *et al*., 2011*a*). OmpD15 is an 88.8 kDa protein (also called Omp85 or Oma87) with homologues among various Gram-negative bacteria including *Haemophilus ducreyi, Pasteurella multocida, H. influenzae, Shigella dysenteriae, Shigella flexneri,* and *Neisseria* spp. (Ruffolo and Adler, 1996; Manning *et al*., 1998; Robb *et al*., 2001; Ayalew *et al*., 2011*b*). Recombinant OmpD15 is immunogenic in mice; however, calves vaccinated with 100 µg in Freund's incomplete antigen developed only minimal antibody responses (Ayalew *et al*., 2011*b*). Unfortunately, challenge data from that study was lost due to a recording machine malfunction (Ayalew and Confer, unpublished data 2010). *M. haemolytica* OmpP2 (41.4 kDa) is a homologue of major outer membrane protein P2 of *Haemophilus influenzae,* which is known to have antigenic variation among *H. influenzae* isolates (Forbes *et al*., 1992; Andersen *et al*., 2003). Both mice and calves vaccinated with recombinant OmpP2 developed low antibody responses suggesting that it may not be highly immunogenic at the dose or in the form that was administered (Ayalew *et al*., 2011*b*).

M. haemolytica *vaccines*

Realistic goals of vaccination

As described above, *M. haemolytica* produces various virulence factors that promote lung colonization, stimulate the production of inflammatory mediators, and enhance evasion of host defense mechanisms, as well as numerous potential immunogens that can stimulate an *M. haemolytica*–specific immune response. In 1975, Thomson *et al.* (1975) demonstrated that on day 1 after shipping cattle that remained healthy had lower numbers of *M. haemolytica* in the nasal cavity and higher concentrations of anti-*M. haemolytica* antibodies than did cattle that became sick. Therefore, vaccination of cattle with efficacious *M. haemolytica* vaccines prior to shipment could potentially reduce shipping fever pneumonia. The overall goals for *M. haemolytica* vaccines are to stimulate an efficacious immune response that would (1) neutralize LKT and kill *Mannheimia haemolytica*, (2) reduce lung colonization, (3) block evasion of host defense mechanisms, and (4) reduce the severity of or prevent pneumonia. The central dogma of *M. haemolytica* vaccination was established by Shewen and Wilkie (1988), whereas they demonstrated that efficacious vaccines must stimulate antibodies against LKT and against surface antigens, although the important surface antigens were not established in that study. Therefore, vaccines against *M. haemolytica* can accomplish this through multiple antibody-mediated mechanisms that (1) neutralize LKT, (2) enhance opsonization and phagocytosis, (3) block adhesion to respiratory cells, and/or (4) augment complement-mediated bacterial killing (Metzger, 2011). It would be an added benefit if *M. haemolytica* vaccines also stimulated protection against multiple serotypes. Cross-serotypic LKT neutralization has been documented (Shewen and Wilkie, 1983*b*; Gentry *et al*., 1988). Surface antigens vary among serotypes making cross-serotypic protection often incomplete, but some degree of cross protection between other serotypes, especially for S1 and S6, has been demonstrated (Gentry *et al*., 1988; Morton *et al*., 1995; Purdy *et al*., 1998; Confer *et al*., 2006; Crouch *et al*., 2012; Zheng *et al*., 2015). In addition, an added potential benefit of efficacious vaccination against bacteria is reduced use of antimicrobials and decreased development of antimicrobial resistance (Jansen *et al*., 2018).

Due to the complexity of BRD, even with efficacy demonstrated in experimental challenge studies, determination of *M. haemolytica* vaccine efficacy can be a daunting task (Table 2). Rice *et al.* (2007) described the difficulties in evaluation of vaccine efficacy field trials in beef calves, and especially the inadequacy of a single field trial to assess vaccine efficacy. For example, review of published vaccine field trials indicated that in some studies, vaccination of cattle with *M. haemolytica* bacterins enhanced protection against shipping fever, whereas in another study, no such protection was noted (Palotay *et al*., 1963; Amstutz *et al*., 1981; Martin, 1983; Perino and Hunsaker, 1997; Larson and Step, 2012). Various experimental challenge

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Table 2. Various *Mannheimia haemolytica* vaccines tested under experimental and field trials

		Efficacy		
Vaccine type	Timeframe	Experimental	Field	Commercially available
Bacterin	Prior to 1990s	Variable	No	No longer
Sodium Salicylate extract	1980s	Variable	Unknown	No
Potassium thiocyanate extract	1980s	Variable	Unknown	No
Saline extract	1980s	Efficacious	Unknown	No
Live – attenuated	1980s	Efficacious	Efficacious or ineffective	No longer
Live – streptomycin-dependent	1985-present	Variable	Efficacious or ineffective	Parenteral or intranasal vaccination
Culture supernatant	1988 to present	Efficacious	Variable	Yes
Bacterin toxoid	1989 to present	Efficacious	Variable	Yes
Capsular polysaccharide	1990s	Variable or poor	Unknown	No
Proprietary extract	1990s	Efficacious	Somewhat efficacious	No longer
Recombinant chimeric protein	2001-present	Partially efficacious	Unknown	No
Ghosts	2003	Efficacious	Unknown	No
Recombinant single protein	2003–3006	Partially efficacious	Unknown	No
LKT-deficient mutant	2012–2013	Efficacious	Unknown	No
Vesicles	2013-present	Efficacious	Unknown	No

methods have been used to assess *M. haemolytica* vaccine efficacy. Originally, licensure of *P. multocida* and *P. haemolytica* bacterins used intraperitoneal vaccination and challenge in mice, which was not a good vaccine model for BRD and was discontinued (Confer, 1993). Several experimental *M. haemolytica* challenge methods for cattle have been used, and the most common ones are aerosol, intratracheal, intrabronchial, or transthoracic routes with *M. haemolytica* alone or in combination with a respiratory virus such as bovine herpesvirus-1 (BHV-1), bovine viral diarrhea virus (BVDV), parainfluenza-3, or bovine respiratory syncytial virus (Frank, 1989). Therefore, comparisons of different experimental vaccine efficacy trials can be confounded by variations in challenge methods. Additional considerations on vaccine trials are: (1) calves persistently infected with BVDV failed to mount an antibody response when vaccinated with a *M. haemolytica* bacterin-toxoid (Fulton *et al.*, 2003), and (2) *M. haemolytica* vaccines are often given with live or killed respiratory viral vaccines, and simultaneous vaccination of BHV-1 seronegative cattle with modified-live BHV-1 vaccine can interfere with the antibody response to *M. haemolytica* (Cortese *et al.*, 2011).

M. haemolytica *bacterins*

For an in-depth review of early vaccines directed against *B. boviseptica*, see Mosier *et al.* (1989*a*) and Mosier (1992). In the early twentieth century, initial attempts to prevent BRD revolved around the use of bacterins, live vaccines, aggressins (bacteria-free inflammatory exudate produced by injection of virulent organisms), and antisera (Mosier *et al.*, 1989*a*). Several early reports indicated that antisera may reduce shipping fever mortality; however, in a later field study, use of prophylactic passive immunization after shipment had little protective value (King *et al.*, 1955). Early vaccine studies often used small groups of cattle, and results suggested that both a bacterin and a live *B. boviseptica* vaccine could probably protect against natural disease; however, injection site abscesses were encountered with live *B. boviseptica* vaccines (Buckley and Gochenour, 1924). In one study, a 3-fold increase in death occurred in bacterin-vaccinated cattle compared with unvaccinated ones (Farley, 1932).

Vaccination of cattle with *M. haemolytica* bacterins via various routes and with various adjuvants stimulated antibodies to the bacterium, and in several experimental studies, bacterins enhanced resistance against challenge, especially when oil adjuvants were used (Carter, 1957; Wohler and Baugh, 1980; Confer *et al.*, 1987; Jericho *et al.*, 1990; Purdy *et al.*, 1997*b*). However, other studies indicated that *M. haemolytica* bacterin-vaccinated cattle were either not protected or had more severe disease than did unvaccinated controls when naturally or experimentally challenged (Hamdy and Trapp, 1964; Hamdy *et al.*, 1965; Schipper and Kelling, 1971; Friend *et al.*, 1977; Wilkie *et al.*, 1980; Confer *et al.*, 1985*b*; Srinand *et al.*, 1995; Frank *et al.*, 1996).

Live M. haemolytica *vaccines*

Interest in and study of live *M. haemolytica* vaccines developed in the 1980s. This was because of data showing inconclusive protection or enhanced disease in bacterin-vaccinated cattle, the discovery of LKT, which is not in bacterins, and demonstration that prior natural exposure of cattle to *M. haemolytica* enhanced resistance against challenge (Thomson *et al.*, 1975; Shewen and Wilkie, 1982, 1983*b*; Confer *et al.*, 1984*b*). Live *M. haemolytica* vaccines studied contained wild type, attenuated, and chemically modified strains as well as a streptomycin-dependent mutant (Srinand *et al.*, 1995; Bowland and Shewen, 2000). In several studies, parenteral or aerosol vaccination with live *M. haemolytica* resulted in enhanced resistance against experimental challenge. However, in field studies, live *M. haemolytica* vaccines either enhanced resistance or had no clear influence on morbidity and mortality (Confer *et al.*, 1983; 1984*a*, 1985*b*, 1986*a*; Kucera *et al.*, 1983; Panciera *et al.*, 1984; Catt *et al.*, 1985; Kadel *et al.*,

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1985; Smith *et al*., 1985; Purdy *et al*., 1986; Blanchard-Channell *et al*., 1987; Srinand *et al*., 1995, 1996*b*). Because LKT is produced primarily during logarithmic phase growth, one study demonstrated that in four of five experiments, aerosol vaccination with live *M. haemolytica* from 6-h cultures enhanced resistance to experimental challenge better than vaccination with 20–22 h live cultures (Confer *et al*., 1984*a*).

An intradermally administered attenuated strain, a chemically modified strain, and the streptomycin-dependent strain were commercialized *M. haemolytica* vaccines (Kucera *et al*., 1983; Henry, 1984; Panciera *et al*., 1984; Confer *et al*., 1984*a*, 1985*b*, 1986*a*; Smith *et al*., 1985). Use of the commercial intradermally administered attenuated *M. haemolytica* vaccine-enhanced resistance in experimentally challenged dairy calves and stimulated significant antibody responses to the bacterial surface in shipped beef calves. However, in one field trial, vaccination afforded marked protection, and in other field trials, the vaccination had no significant effect on performance, morbidity, or mortality (Confer *et al*., 1983; Henry, 1984; Smith *et al*., 1985; Purdy *et al*., 1986). The intradermal vaccine was later removed from the market partially because of difficulty in administration and local injection site reactions.

In one field trial, experimental live *M. haemolytica* vaccination by aerosol or subcutaneous routes prior to shipment stimulated significant antibody responses to the bacterial surface; however, significant protection against BRD was not demonstrated (Confer *et al*., 1983).

Calves vaccinated with the chemically modified *M. haemolytica* strain had increased resistance to a BHV-1/*M. haemolytica* challenge when compared with nonvaccinated controls (Kucera *et al*., 1983). Later, 11 cases of systemic *M. haemolytica* infection were described in post-vaccinated calves. Those calves had meningitis, polyarthritis, and dermatitis and/or cellulitis, and the infecting bacterial strain was identified by restriction endonuclease analysis as the chemically modified vaccine strain (Zeman *et al*., 1993). That vaccine is no longer available.

Genetically modified *M. haemolytica* have been described including streptomycin-dependent, AroA deletion, and LKT – modified mutants. AroA is a component a metabolic pathway important in aromatic amino acid synthesis, construction of several AroA deletion mutants of *M. haemolytica* have been described (Homchampa *et al*., 1994; Tatum *et al*., 1994; Tatum and Briggs, 2005). In one study, vaccination of mice with live *M. haemolytica* *aroA*$^{-}$ mutants reduced death in vaccinated mice; however, to our knowledge cattle studies have not been done (Homchampa *et al*., 1994).

A bivalent vaccine containing streptomycin-dependent *M. haemolytica* and *P. multocida* mutants has been approved for parenteral or (recently) intranasal vaccination and marketed for many years (OncePMH®). (Catt *et al*., 1985; Kadel *et al*., 1985; Blanchard-Channell *et al*., 1987; Chengappa *et al*., 1989; Mosier *et al*., 1998). Two studies demonstrated significant increases in antibodies to *M. haemolytica* following vaccination with the mutant bacteria as well as reduced clinical signs and lesions following BHV-1/*M. haemolytica* challenge (Catt *et al*., 1985; Blanchard-Channell *et al*., 1987). Greater economic gains in vaccinated, non-preconditioned cattle were noted following a 50-day field trial (Kadel *et al*., 1985). Vaccination stimulated significant increases in antibodies to CPS and whole bacteria after one dose, whereas significant increases in antibodies against LKT and IROMPs required a booster vaccination (Srinand *et al*., 1996*a*). In other studies, the streptomycin-dependent vaccine stimulated antibodies to *M. haemolytica* cell surface but not against LKT, and clinical and lesion scores for vaccinates were not significantly less than those in control cattle following transthoracic or intrabronchial *M. haemolytica* challenge (Srinand *et al*., 1996*b*; Mosier *et al*., 1998). Vaccinated veal calves had reduced respiratory morbidity compared with non-vaccinates (Schnepper *et al*., 1996).Vaccination of 14–20-day-old Holstein calves with the vaccine stimulated significant increases in antibodies; however, differences between vaccinated and control calves were not seen in BRD treatment data (Aubry *et al*., 2001). Recently, a study compared cattle vaccinated with the streptomycin-dependent mutant vaccines by intranasal or subcutaneous routes and found no differences in cattle performance due to the route of vaccination (Spore *et al*., 2017).

LKT-deficient *M. haemolytica* mutants have been studied as potential vaccines. Frank *et al*. demonstrated that intranasal exposure of shipped calves to live *M. haemolytica* with a 1-kb deletion in the *lktA* gene resulted in increased serum *M. haemolytica* antibodies and decreased *M. haemolytica* nasal colonization (Frank *et al*., 2003). Recently, oral or parenteral vaccination of calves, sheep, and goats with live *M. haemolytica* mutants that produced N-terminal truncated LKT, which retained the neutralizing epitope, enhanced resistance against *M. haemolytica* challenge and stimulated both hemagglutinating and LKT-neutralizing antibodies (Briggs *et al*., 2012, 2013).

M. haemolytica *extract vaccines*

With the realization that *M. haemolytica* bacterins either failed to protect or caused enhanced disease, various antigen extraction procedures, including saline, potassium thiocyanate, and sodium salicylate, were studied to try to develop a better vaccine (Durham *et al*., 1986).

Matsumoto *et al*. (1984) used a 2.5% saline extraction of *M. haemolytica* adsorbed to aluminum hydroxide gel as subcutaneous vaccine and extract alone for aerosol vaccination. They demonstrated enhanced resistance against a BHV-1/*M. haemolytica* challenge after subcutaneous vaccination; however, aerosol vaccination resulted in inconsistent results. Warm saline extraction of logarithmic-phase bacteria removed capsule and multiple surface proteins (Gentry *et al*., 1982; Confer et al., 1985*a*; Lessley *et al*., 1985; McKinney *et al*., 1985). Vaccination with that saline extract enhanced resistance against transthoracic challenge, and there was a significant correlation between high antibodies to various protein components and low lesion scores (Confer *et al*., 1985*a*; McKinney *et al*., 1985). Likewise, vaccination of cattle with a carbohydrate-protein subunit made by chromatofocusing of *M. haemolytica* saline extract enhanced resistance against transthoracic challenge (Confer *et al*., 1989).

Sodium salicylate extraction of *M. haemolytica* S1 and S6 resulted in similar SDS-PAGE profiles as well as protein, carbohydrate, lipid, and phosphorus compositions (Donachie *et al*., 1984). Vaccination with salicylate extracts with aluminum hydroxide adjuvant enhanced the resistance of calves and lambs against challenge with homologous serotypes (Gilmour *et al*., 1982, 1983). In a later study, however, vaccination with salicylate extract failed to provide protection of calves against intranasal and intratracheal challenge and may have actually enhanced disease (Gilmour *et al*., 1987). Vaccination of calves with a salicylate extract containing IROMPs stimulated significant antibody responses to capsular polysaccharide and IROMPS, and those calves had significantly lower percent lung lesions than did controls after experimental challenge (Sreevatsan *et al*., 1996).

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Potassium thiocyanate extracts of *M. haemolytica* as vaccines were studied briefly. Vaccination of mice and hamsters with *M. haemolytica* saline extract, potassium thiocyanate extract or bacterins indicated potassium thiocyanate extract resulted in greatest resistance against challenge (Tadayon and Lauerman, 1981). Immunization of mice with a potassium thiocyanate extract of *M. haemolytica* enhanced cross-protection against *P. multocida* challenge (Mukkur, 1977). Vaccination of calves via intranasal, subcutaneous, or intramuscular routes resulted in variable degrees of protection against aerosol BHV-1/*M. haemolytica* challenge (Yates *et al*., 1983). Parenteral vaccination enhanced resistance and reduced bacterial isolation at necropsy better than did aerosol vaccination.

Capsular polysaccharide extract vaccines were tested experimentally. Vaccination of cattle with purified capsular polysaccharide stimulated antibodies to the capsule, and the intensity of the response and immunoglobulin type produced were dependent on the adjuvant used (Tigges and Loan, 1993). In another study, vaccination of calves with capsular polysaccharide alone or in conjunction with *M. haemolytica* culture supernatant or recombinant LKT did not protect calves (Conlon and Shewen, 1993). In fact, capsular polysaccharide vaccination was associated with a 36% incidence of anaphylaxis. Others demonstrated that vaccination of calves with capsular polysaccharide with various dosages of muramyl dipeptide analogs stimulated resistance against challenge in several experiments; however, in an experiment comparing the capsular vaccine against commercial vaccines, the capsular vaccine had little efficacy (Brogden *et al*., 1995). More recently, authors suggested that (2→8)-α-Neu5Ac, which is a component of the capsule of Group B *Neisseria meningitidis, E. coli* K1, and *M. haemolytica* S2, might be used as a component of a conjugate vaccine (Robbins *et al*., 2011).

A commercial vaccine that was a mild detergent extract of *M. haemolytica* was marketed for several years (Septimune® PH-K). The extraction method and detergent used were proprietary and not available in the literature. In published studies, the vaccine stimulated variable antibody responses to cell surface antigens and low anti-LKT antibodies (Confer and Panciera, 1994; Srinand *et al*., 1996*a*; Confer *et al*., 1998). In two studies, Septimune enhanced resistance against *M. haemolytica* challenge, and in one study, vaccinated, shipped cattle had better, though not statistically significant, performance and health than did nonvaccinated cattle (Brogden *et al*., 1989; Hill *et al*., 1993; Confer and Panciera, 1994). That vaccine was later removed from the market. Saline, sodium salicylate, and potassium thiocyanate extracts likely lacked appreciable quantities of LKT to stimulate strong immunity and were not studied beyond the 1990s.

LKT supernatant vaccines

With the discovery of LKT secretion into culture supernatants and correlation between high LKT-neutralizing antibodies and resistance against the field or experimental BRD, Dr Bruce Wilkie's laboratory began to study the use of culture supernatant as a vaccine (Gentry *et al*., 1985; Shewen and Wilkie, 1983*a*, 1983*b*). Besides LKT, *M. haemolytica* culture supernatant contains numerous secreted and surface antigenic proteins as well as capsular polysaccharide and LPS (Mosier *et al*., 1994; Mellors and Lo, 1995; Ayalew *et al*., 2017*b*).

Shewen and Wilkie (1988) demonstrated that vaccination with *M. haemolytica* S1 LKT-rich culture supernatant enhanced resistance against intrabronchial challenge with the homologous serotype, which led to the licensure of the commercial culture supernatant vaccine Presponse®. In that study, vaccination with LKT-rich culture supernatant derived from *P. haemolytica* S11 (now *M. glucosida*) stimulated LKT neutralizing antibodies but was not as efficacious against *M. haemolytica* S1 challenge. Those data led to the conclusion that antibodies to both LKT and surface antigens are important for enhancing resistance against *M. haemolytica* pneumonia. In addition, they noted that two doses of vaccine were more efficacious than one; however, in a 1995 study, one dose of the commercial vaccine was as efficacious as two doses against an intrabronchial challenge leading to licensure of Presponse for one-dose protection (Conlon *et al*., 1995). The rationale for one-dose protection is based on a spontaneous rise in anti-*M. haemolytica* antibodies in young calves due to natural exposure through nasopharyngeal colonization; therefore, natural exposure is equivalent to primary vaccination (Hodgins and Shewen, 1998; Prado *et al*., 2006). In another study, two doses of Presponse stimulated low peak antibody responses in 2-week-old, colostrum-deprived dairy calves; however, vaccinated calves had significant reductions in clinical signs and lesion scores compared with placebo-vaccinated calves (Hodgins and Shewen, 2000). In addition, Presponse vaccination with two doses of vaccine stimulated significant antibodies to surface antigens and to LKT, and vaccinated calves had a significant reduction in lung lesions after challenge when compared with controls (Sreevatsan *et al*., 1996). In the previously cited culture supernatant vaccine studies, anti-LKT and anti-surface antigen antibodies were demonstrated after vaccination. In contrast, Srinand *et al*. (1996*a*) demonstrated antibody responses to capsular polysaccharide but low to no anti-LKT or anti-whole cell antibodies in calves following Presponse vaccination. Similarly, we demonstrated that Presponse stimulated significant increases in antibodies to *M. haemolytica* whole cells and LKT as early as 7–14 days after vaccination; however, those responses were often lower than seen with other LKT-containing vaccines (Confer *et al*., 1998, 2001, 2003).

As with many commercial vaccines, published studies of field trials are not numerous, and results can be variable due to conditions of the study, type of cattle used, and which respiratory pathogens may be involved in causing clinical disease. Bateman (1988) vaccinated recently shipped, non-preconditioned calves with Presponse and found a slight decrease in morbidly, a slight improvement in treatment responses, and reduction in relapses. Jim *et al*. (1988) demonstrated reduced mortality, increased response to treatment, and economic benefits in feedlot calves vaccinated with Presponse In another study, Presponse vaccination of auction calves reduced relapse rates and mortality; however, vaccinated calves from ranches had no changes in morbidity rates or weight gains compared with nonvaccinated calves (Thorlakson *et al*., 1990). Average daily gains were improved in cattle vaccinated with Presponse at time of receiving compared with control cattle; however, morbidity and mortality data were not significantly different between vaccinated and nonvaccinated cattle (McLean *et al*., 1990). Brazle (1992) vaccinated steers and bull calves and found no difference between vaccinated and controls with respect to weight gain, mortality, or morbidity. Fewer treatments, however, were required among vaccinates compared with controls. Malcolm-Callis *et al*. (1994) demonstrated no benefit to vaccination in low morbidity calves but found increased gains and reduced treatments in vaccinated stressed calves. Bechtol and Jones (1996) suggested that Presponse vaccination of lightweight calves in a backgrounding lot was economically beneficial. Ives *et al*. (1999) found vaccination of calves with

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Presponse plus modified viral vaccines tended to reduce BRD incidence and retreatment rates, but those differences were not significant at $P < 0.05$.

In several experimental studies, Presponse was supplemented with either recombinant LKT, purified capsular polysaccharide, recombinant sialoglycoprotease, or recombinant outer membrane lipoprotein PlpE (Conlon *et al*., 1991; Conlon and Shewen, 1993; Confer *et al*., 2003, 2006; Shewen *et al*., 2003). In those studies, the addition of any of the three recombinant proteins reduced clinical disease and/or lesion scores compared with Presponse alone, whereas addition of capsular polysaccharide failed to enhance resistance against challenge. In one experimental study, *M. haemolytica* culture supernatant was incorporated into polymerized methacrylic acid hydrogels and orally administered to calves (Bowersock *et al*., 1994). Vaccinated calves had less severe lung lesions and lived longer after intrabronchial challenge.

Bacterin-toxoid vaccines

Combinations of culture supernatants and killed *M. haemolytica* are marketed as bacterin-toxoid vaccines. Common examples include One Shot® and Pulmo-Guard®, and bacterin-toxoids are highly immunogenic stimulating intense antibody responses to surface antigens and LKT. One study demonstrated that One Shot vaccination also stimulated the production of the acute-phase protein haptoglobin (Arthington *et al*., 2013; Moriel and Arthington, 2013). Srinand *et al*. (1996*a*) demonstrated that a commercial bacterin-toxoid stimulated a significant antibody response to LKT, capsular polysaccharide, and whole cells but not to IROMPs. In numerous studies, high antibody responses to whole *M. haemolytica* and LKT were demonstrated after vaccination of cattle with One Shot or Pulmo-Guard (Loan and Tigges, 1989; Confer *et al*., 1998; 2001, 2003, 2006; Mosier *et al*., 1998; Frank *et al*., 2002; Fulton *et al*., 2003; Ayalew *et al*., 2004; Bowersock *et al*., 2014).

Parenteral vaccination of cattle with *M. haemolytica* bacterin-toxoid vaccines enhanced resistance against experimental intrabronchial or transthoracic *M. haemolytica* S1 challenge and often-enhanced resistance more than other vaccines with which it was compared (Loan and Tigges, 1989; Confer and Panciera, 1994; Srinand *et al*., 1996*a*; Mosier *et al*., 1998). Cattle vaccinated with One Shot, a bacterin-toxoid derived from *M. haemolytica* S1, had 46% reduction in lesion scores after challenge with *M. haemolytica* S6 when compared with control lesion scores (Confer *et al*., 2006). In that study, cattle vaccinated with One Shot supplemented with recombinant outer membrane lipoprotein PlpE had a 62% reduction in lesion scores compared with control cattle. Because of the relatedness between *M. haemolytica* and *B. trehalosi*. Bowersock *et al*. (2014) demonstrated that vaccination of calves with a multivalent modified-live virus vaccine containing One Shot enhanced resistance against an intrabronchial *B. trehalosi* challenge compared with vaccination with the virus vaccine alone.

There have been fewer published field trials with bacterin-toxoid vaccines compared with culture supernatant vaccine trials. Vaccination of cattle on entry to a feedlot with a *H. somni – M. haemolytica* bacterin-toxoid (SOMNU-STAR Ph, Elanco, Canada) resulted in increased antibodies to both bacteria and reduced morbidity (Van Donkersgoed *et al*., 1993). Frank *et al*. (2002) found that *M. haemolytica* bacterin-toxoid vaccination prior to shipping did not enhance the resistance of cattle treated with florfenicol at an entry to the feedlot compared with nonvaccinated, florfenicol-treated calves. In field trials, Wildman et al. (2008) recommended the use of a bacterin-toxoid in conjunction with modified-live viral vaccines in a vaccination program for feedlot cattle. Vaccination of cattle with Pulmo-guard on arrival at a feedlot, significantly reduced mortality, but morbidity and average daily gain were unaffected by vaccination (MacGregor *et al*., 2003).

Autogenous vaccines

Several companies will make an *M. haemolytica* vaccine from a bacterial strain isolated from a specific farm, ranch, feedlot, or dairy. That vaccine is to be used only on those premises, and herd-specific vaccines have been used against several cattle pathogens (Attia *et al*., 2013). Although these vaccines are not subject to the traditional safety and efficacy studies required for licensure of a commercial vaccine, they are required to be manufactured in a licensed facility and are subject to various regulations and guidelines for use that vary among countries (Attia *et al*., 2013) (https://www.aphis.usda.gov/animal_health/vet_biologics/publications/pel_4_16.pdf). Published efficacy data on *M. haemolytica* autogenous vaccines are limited to an intraperitoneal *M. haemolytica* autogenous vaccine used effectively to control mastitis in sheep (Kabay and Ellis, 1989). *M. haemolytica* autogenous vaccines are used in the field for BRD control; however, we and others have been unable to find published controlled studies of the use of *M. haemolytica* autogenous vaccines in BRD, and vaccine efficacies remain unknown (Miles and Rogers, 2014).

Experimental M. haemolytica *vaccines*

In recent years, several experimental approaches have been reported in attempts to develop improved *M. haemolytica* vaccines. These include outer membrane vesicles, recombinant proteins including chimeric proteins, and bacterial ghosts.

Gram-negative bacteria produce closed outer membrane blebs that detach as vesicles, which contain OMPs, LPS, periplasmic proteins, peptidoglycans, and secretory components such as toxins and have been studied as non-living, acellular vaccines against several bacteria (Kuehn and Kesty, 2005; Koeberling *et al*., 2011; Nieves *et al*., 2011; Park *et al*., 2011). *M. haemolytica* spontaneously produces vesicles *in vitro,* and proteomic analyses revealed 58 proteins of outer membrane or periplasmic membrane origin and LKT (Ayalew *et al*., 2013). Similarly, differences were minimal between *M. haemolytica* vesicle protein profiles and outer membrane protein profiles, and intranasal immunization of mice stimulated serum IgA and IgG1 antibody responses that reacted with SSA-1, OmpA, OMP P2, and several unidentified antigens (Roier *et al*., 2013). Ramirez Rico *et al*. (2017) demonstrated that the culture supernatant of *M. haemolytica* S2 had higher protease activity than did outer membrane vesicles. Vaccination of calves with *M. haemolytica* vesicles stimulated antibodies to LKT and to surface antigens, and vaccinated calves had significant reductions in clinical signs and lesion scores after intrabronchial challenge (Ayalew *et al*., 2013).

As described in previous sections, addition of recombinant LKT, sialoglycoprotease, or PlpE to commercial vaccines enhanced resistance against experimental challenge compared with the vaccine alone (Conlon *et al*., 1991; Confer *et al*., 2003, 2006; Shewen *et al*., 2003). In each of those studies, vaccination of calves with only the recombinant protein demonstrated little or some beneficial effects, at least at the dosage given and with the adjuvant used. Feeding cattle with dried alfalfa expressing truncated LKT resulted in transient nasal IgA anti-LKT antibodies, and in a small pilot study, two orally vaccinated calves challenged with *M. haemolytica* had no lung lesions, whereas the two nonvaccinated controls had 11 and 27% pneumonic lesions (Shewen *et al*., 2009).

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In several studies, recombinant chimeric or fusion proteins have been produced using the neutralizing epitope fragment of *lktA* and an immunogenic protein expressed from another gene. A recombinant protein with glutathione-S-transferase (GST), neutralizing epitope of LKT and *Bordetella bronchiseptica* fimbrial protein stimulated higher anti-LKT antibody responses in mice than did the GST-LKT protein minus fimbrial protein (Rajeev *et al*., 2001). We produced several chimeric proteins from the neutralizing epitope of LKT and the major epitope of PlpE and demonstrated that mice vaccinated with those proteins developed antibodies against PlpE and LKT that had both complement-mediated bacterial killing and LKT neutralization activities (Ayalew *et al*., 2004, 2008). Subcutaneous vaccination of cattle with a PlpE-LKT chimeric (SAC89) plus formalin- killed bacteria in an oil-in-water adjuvant resulted in 75% lower lesion scores compared with controls, whereas vaccination with the chimeric protein or bacterin alone resulted in approximately 35% lower lesion scores compared with controls (Confer *et al*., 2009*a*). Subsequently, intranasal vaccination of cattle with PlpE-LKT chimeric protein plus cholera toxin stimulated nasal anti-whole cell and anti-LKT antibodies, whereas intranasal vaccination of calves with a PlpE-LKT-cholera toxin subunit B chimeric protein (SAC102) stimulated serum and nasal antibodies (Ayalew *et al*., 2009; Confer *et al*., 2009*b*). Calves vaccinated with SAC102 had lower clinical signs after intrabronchial challenge than did non-vaccinated calves. Recently, a similar PlpE-LKT chimeric protein stimulated anti-PlpE and anti-LKT antibodies in mice (Batra *et al*., 2016*b*). Those scientists intranasally vaccinated Bighorn sheep with a recombinant BHV-1-vectored vaccine expressing PlpE-LKT chimeric proteins. Vaccinated sheep developed anti-LKT antibodies, but inconsistently developed anti-surface antibodies, and the vaccine failed to protect against *M. haemolytica* challenge (Batra *et al*., 2017).

Bacterial ghosts are a non-living vaccine strategy, wherein bacteria are infected with a temperature-controlled lytic phage that causes membrane tunnels through which the bacterial genome (cytosol?) is expelled leaving a bacterial envelop (ghost) that has proteins that were not modified by exposure to formalin or another bactericidal substance, as with a bacterin (Szostak *et al*., 1996; Lubitz *et al*., 1999). Vaccination of mice and calves with *M. haemolytica* ghosts plus adjuvant enhanced resistance against challenge similar to a commercial vaccine (Pastobov®, Merial) (Marchart *et al*., 2003*a*, 2003*b*).

Conclusions

In the last 30 years, much has been learned about immunogens and potential immunogens of *M. haemolytica*. Current commercial vaccines, in general, are improvements over prior bacterins, albeit field trials have not always demonstrated efficacy in the face of a complex of pathogens and environmental stressors. Enough data have been generated on immunogenic recombinant *M. haemolytica* proteins that warrant further studies to develop a new generation of *M. haemolytica* vaccines with increased efficacy beyond that experienced with today's vaccines.

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