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LKTA and PlpE small fragments fusion protein protect against *Mannheimia haemolytica* challenge

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ABSTRACT

Bovine respiratory disease (BRD) complex is a major cause of economic losses for the cattle backgrounding and feedlot industries. *Mannheimia haemolytica* is considered the most important pathogen associated with this disease. Vaccines against *M. haemolytica* have been prepared and used for many decades, but traditional bacterins have failed to demonstrate effective protection and their use has often exacerbated disease in vaccinated animals. Thus, the BRD complex continues to exert a strong adverse effect on the health and wellbeing of stocker and feeder cattle. Therefore, generation of recombinant proteins has been helpful in formulating enhanced vaccines against *M. haemolytica*, which could confer better protection against BRD. In the present study, we formulated a vaccine preparation enriched with recombinant small fragments of leukotoxin A (LKTA) and outer-membrane lipoprotein (PlpE) proteins, and demonstrated its ability to generate high antibody titers in rabbits and sheep, which protected against *M. haemolytica* bacterial challenge in mice.

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1. Introduction

Bovine respiratory disease (BRD) complex is a major cause of economic losses for the cattle backgrounding and feedlot industries (Gagea et al., 2006; Snowder et al., 2006). BRD research has provided significant understanding of the disease over the past 30 years (Fulton, 2009). Modern research tools have been used to generate advances in products including vaccines as well as technological, biological, and pharmacological developments. The bacterial component of the BRD complex continues to have a major adverse effect on health and wellbeing of stocker and feeder cattle (McVey, 2009; Griffin, 2010).

BRD involves complex interactions among viral and bacterial pathogens that can lead to intense pulmonary inflammation (fibrinous pleuropneumonia) (Czuprynski, 2009). Among bacterial pathogens associated with the disease, two Gram negative bacteria are the more relevant: *Histophilus somni*, which causes respiratory disease, septicemia, thrombotic meningoencephalitis, myocarditis, arthritis, and abortion (Corbeil, 2007; O'Toole et al., 2009; Sandal and Inzana, 2010), and *Mannheimia haemolytica*, which is considered the most important (Confer, 2009). Pathogens involved in the BRD complex have developed intricate mechanisms to thwart both the innate and adaptive immune responses of their hosts. These immune evasion strategies are likely to contribute to the failure of currently available vaccines to provide complete protection to cattle against these pathogens (Srikumaran et al., 2007).

M. haemolytica is an opportunist bacterium, gaining access to the lungs, when host defenses are compromised by stress or infection with respiratory viruses or mycoplasma. Although, several serotypes act as commensals, A1 and A6 are the most common isolates from pneumonic lungs (Rice et al., 2007; Confer, 2009).

Among potential virulence factors of *M. haemolytica*, leukotoxin A (LKTA), an exotoxin that is cytolytic for macrophages, neutrophils and all other leukocyte subsets (Berggren et al., 1981; Shewen and Wilkie, 1982), is critical in the induction of pneumonia (Jeyaseelan et al., 2002; Rice et al., 2007; Czuprynski, 2009) and antibodies

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against it are needed to generate immunity against BRD (Shewen and Wilkie, 1982). Protein LKTA (GenBank M20730) has 953 amino acids and a molecular mass of 104 kDa (Fig. 1). It is a member of the repeats-in-toxin (RTX) family of multidomain exotoxins (Lo, 1990) and contains several highly conserved glycine-rich repeats of nine amino acids near the C-terminal end of its structure (Lo, 1990; Coote, 1992).

Another immunogenically relevant protein is a 45-kDa outer-membrane lipoprotein, PlpE (Pandher et al., 1999; Ayalew et al., 2004; GenBank ABB20693.1; Fig. 2). It has been demonstrated that when PlpE is added to the vaccine formulation, the vaccinated cattle have significantly greater resistance against experimental challenge (Confer et al., 2003, 2006, 2009a,b).

Recently, Confer et al. (2009 a,b) showed in a relevant work series that the PlpE–LKTA chimeric protein, in combination with a bacterin of *M. haemolytica*, stimulated significant protection against a severe transthoracic challenge with the bacterium (Ayalew et al., 2008; Confer et al., 2009a,b). In this case, they included the epitope involved in neutralization of LKTA (localized at 146-amino acid region of the C-terminus of native LKTA, Fig. 1) in the chimeric protein (Lainson et al., 1996; Rajeev et al., 2001). To complement these investigations, we show in the present work that a recombinant fragment of LKTA (it includes five haemolysin binding domains also) that constitutes a neutralization epitope, even its first 46 amino acids (one third), mixed with a fragment of PlpE, also protected against the bacterial challenge. In addition, we used aluminum hydroxide [$Al(OH)_3$] as the adjuvant in vaccine preparation because it is a well-documented adjuvant for stimulating immunity (Exley et al., 2010) by potentiating the immune response (Seubert et al., 2008). It was used instead of the cholera toxin or Freund's incomplete adjuvant, which were used as adjuvants in other studies.

Vaccination aims to mimic development of naturally acquired immunity by inoculation of immunogenic components of the pathogen in question (Meeusen et al., 2007) and has been shown to be the single most efficient means of preventing bacterial, viral and parasitic infections (Potter et al., 2008). Vaccines have been used for many decades, but traditional bacterins have failed to demonstrate effective protection and their use has often exacerbated disease in vaccinated animals (Rice et al., 2007). Modern-day vaccines preferentially use culture supernatants containing LKTA and other soluble antigens. Nevertheless, these vaccines have 50–70% efficacy in preventing *M. haemolytica* pneumonia (Rice et al., 2007) and can cause severe adverse effects. Therefore, more effective control of *M. haemolytica* pneumonia requires a combination of more precise diagnosis, efficacious vaccines, therapeutic intervention and improved management practices. Therefore, generation of recombinant proteins is helpful in formulating recombinant vaccines against *M. haemolytica*, which could confer better protection against BRD.

2. Material and methods

2.1. Bacterial strains and culture conditions

Escherichia coli TOP10 or M15 (Invitrogen) was used as the host for cloning and propagation of plasmids, which were cultured in Luria–Bertani (LB) broth supplemented with thymine (50 mg/ml) and ampicillin (100 mg/ml), chloramphenicol (25 mg/ml), or kanamycin (50 mg/ml), as necessary. *M. haemolytica A1* (ATCC: 43270) was used to obtain bacterial DNA for the PCR.

2.2. Extraction and quantification of genomic DNA and recombinant methods

Bacterial genomic DNA was obtained by Illustra bacteria genomic Prep Mini Spin kit (GE Healthcare). DNA integrity was routinely evaluated by electrophoresis in agarose gels stained with Safe DNA gel stain (Invitrogen) and quality evaluated by A_{260}/A_{280} absorbance. All DNA cloning and ligation were carried out using standard recombinant DNA techniques (Ausubel et al., 2001; Sambrook et al., 2001).

2.3. Oligonucleotide design and PCR

LKTA is a soluble secreted and highly toxic protein. As attempts to study immunogenicity of its carboxyl-terminal region (that includes the RTX domain) have not been successful (Lainson et al., 1996), we decided to produce a fusion protein with a fragment of LKTA from amino acid 573 to 845 to obtain antibodies against it (Fig. 1). This 273-amino acid polypeptide includes five repeated hemolysin-type calcium-binding domains, which are highly conserved sequence regions among different serotypes. For the PlpE construct, we decided to eliminate first 18 amino acids, which encodes for a signaling peptide, and our construct therefore included remaining 338 amino acids (Fig. 2).

Oligonucleotide sequences of the primers used for PCR were as follows: for *lktA* gene, the sense primer 5′-GAAAAGGCCTGATG GTGCAGCAAGTTCTAC-3′ and the antisense primer 5′-GGCACAAGC TTACGAAATCAGCCTCTCGG-3′ were used to amplify an 846-bp fragment that encoded for 273-amino acid fragment of the LKTA. For *plpE* gene, the sense primer 5′-AATAGGCCTGCGGAGGAAGCGG-TAGC-3′ and the antisense primer 5′-ATAAGCTTATTTTTTCTCGCTA ACCATTA-3′ were used to amplify a 1014-bp fragment that encoded for 338 amino acids of PlpE. Primers were designed with enzyme restriction sites introduced into each. *Stu*I site was introduced in both forward primers and *Hind*III in both reverse primers in order to achieve directional cloning in the expression vector.

PCR was carried out in a Perkin-Elmer GeneAmp PCR System 2400 Thermocycler (Perkin-Elmer, Foster City, California). For all

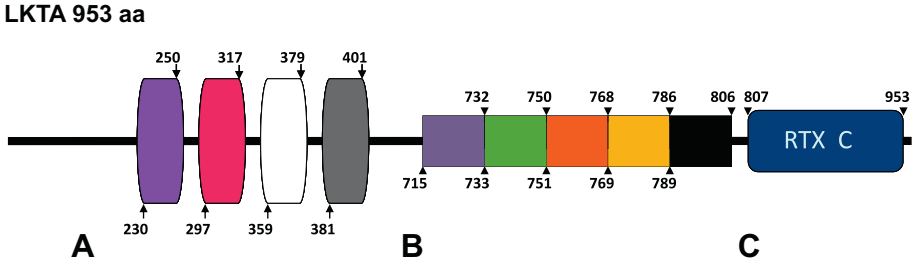

Fig. 1. LKTA protein domain structure, which is 953 amino acids long. (A) Possible transmembrane domains, (B) five repeated hemolysin-type calcium-binding regions, and (C) RTX domain at the carboxyl-terminal region. Numbers indicate amino acid position. Sequence was obtained from UniProt sequence (accession number Q9EV32) and analyses were performed based on the software by Hau et al. (2007).

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