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A chimeric protein comprising the immunogenic domains of *Mannheimia haemolytica* leukotoxin and outer membrane protein PlpE induces antibodies against leukotoxin and PlpE

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ABSTRACT

Mannheimia haemolytica is a very important pathogen of pneumonia in ruminants. Bighorn sheep (BHS, *Ovis canadensis*) are highly susceptible to *M. haemolytica*-caused pneumonia which has significantly contributed to the drastic decline of bighorn sheep population in North America. Pneumonia outbreaks in wild BHS can cause mortality as high as 90%. Leukotoxin is the critical virulence factor of *M. haemolytica*. In a 'proof of concept' study, an experimental vaccine containing leukotoxin and surface antigens of *M. haemolytica* developed by us induced 100% protection of BHS, but required multiple booster injections. Vaccination of wild BHS is difficult. But they can be vaccinated at the time of transplantation into a new habitat. Administration of booster doses, however, is impossible. Therefore, a vaccine that does not require booster doses is necessary to immunize BHS against *M. haemolytica* pneumonia. Herpesviruses are ideal vectors for development of such a vaccine because of their ability to undergo latency with subsequent reactivation. As the first step towards developing a herpesvirus-vectored vaccine, we constructed a chimeric protein comprising the leukotoxin-neutralizing epitopes and the immuno-dominant epitopes of the outer membrane protein PlpE. The chimeric protein was efficiently expressed in primary BHS lung cells. The immunogenicity of the chimeric protein was evaluated in mice before inoculating BHS. Mice immunized with the chimeric protein developed antibodies against *M. haemolytica* leukotoxin and PlpE. More importantly, the anti-leukotoxin antibodies effectively neutralized leukotoxin-induced cytotoxicity. Taken together, these results represent the successful completion of the first step towards developing a herpesvirus-vectored vaccine for controlling *M. haemolytica* pneumonia in BHS, and possibly other ruminants.

1. Introduction

Mannheimia haemolytica is a very important pathogen of pneumonia in ruminants (Mosier, 1997). Of all the ruminants, bighorn sheep (BHS, *Ovis canadensis*) are the most susceptible species (Dassanayake et al., 2009). Pneumonia is the primary disease responsible for the drastic decline of BHS population in North America from an estimated two million in the 1800's to less than 70,000 at the present time (Valdez and Krausman, 1999). *M. haemolytica* is frequently detected in the lungs of pneumonic BHS from the wild (Shanthalingam et al., 2014). Experimental inoculation of BHS with *M. haemolytica* consistently results in 100% mortality (Onderka and Wishart, 1988; Foreyt et al., 1994; Dassanayake et al., 2009). Pneumonia outbreaks in wild BHS herds can cause initial all age mortality as high as 90% (Clifford et al., 2009). Following recovery, the herd continues to experience lamb mortality for several years (Coggins, 1988; Coggins and Matthews, 1992; Ryder et al., 1992). Current management strategies to replenish the affected herds involve transplantation of BHS from healthy herds. Despite a large number of transplantations over the decades, the BHS numbers remain low because of recurrent pneumonia outbreaks. BHS vaccinated against the pneumonia pathogens are more likely to survive in the new habitat than un-vaccinated BHS.

In a 'proof of concept' study, an experimental vaccine against *M. haemolytica* developed by us induced 100% protection in captive BHS. This vaccine, however, required multiple booster injections

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(Subramaniam et al., 2011). Vaccination of wild BHS is difficult. Administration of booster doses is even more difficult, if not impossible. During transplantation operations, however, BHS are in captivity for a day or two, and hence can readily be vaccinated, prior to their release into the new habitat. Thus it is possible to vaccinate and protect BHS against *M. haemolytica* if a vaccine that does not require administration of booster doses is available.

Virus-vectored vaccines against several bacterial and viral pathogens have been developed (Draper and Heeney, 2010). Herpesviruses are ideal vectors for the insertion of heterologous antigens because of their large genomes. More importantly, their ability to undergo latency with subsequent reactivation obviates the need for booster doses since the heterologous antigens are also expressed during lytic infection following reactivation, when the animals come under stress.

M. haemolytica produces several virulence factors including the capsule, outer membrane proteins, lipopolysaccharide and leukotoxin (Lkt). Based on the fact that Lkt-deletion mutants do not cause mortality in BHS (Dassanayake et al., 2009), Lkt has been recognized as the critical virulence factor in the pathogenesis of *M. haemolytica* infection in BHS. Lkt belongs to the RTX (repeats-in-toxin) family of exotoxins produced by a group of Gram-negative bacteria (Linhartová et al., 2010). Although *M. haemolytica* Lkt is cytolytic to all subsets of ruminant leukocytes, polymorphonuclear leukocytes (PMNs) are the most susceptible subset. Lkt-induced lysis of PMNs is the primary cause of acute inflammation and lung injury characteristic of *M. haemolytica*-caused pneumonia (Jeyaseelan et al., 2002). Previously we have shown that induction of antibodies to the Lkt-neutralizing epitopes and to the surface antigens of *M. haemolytica* can protect BHS against virulent *M. haemolytica* challenge (Subramaniam et al., 2011). The outer membrane phospho-lipoprotein PlpE, is surface-exposed and present in all serotypes of *M. haemolytica* (Pandher et al., 1998, 1999). Cattle vaccinated with recombinant PlpE developed significant IgG response to the whole bacterial cell and had significantly lower lesion scores when compared to the un-vaccinated controls following infection (Confer et al., 2003). Our long term goal is to develop a herpesvirus-vectored vaccine expressing protective immunogens of *M. haemolytica*. In order to accomplish that goal, it is imperative to first evaluate the immunogenicity of *M. haemolytica* immunogens expressed in mammalian cells which are quite distinct from those expressed in prokaryotes. Therefore, as the first step in this direction, we developed a chimeric protein comprising the immunogenic domains of Lkt and the outer membrane protein PlpE, and determined its immunogenicity in mice.

2. Materials and methods

2.1. Bacteria and cells

M. haemolytica serotype A2 strain, WSU-1 (Foreyt et al., 1994), isolated from the naso-pharynx of a domestic sheep, was used in this study. Bacteria were grown in brain heart infusion (BHI; Remel, Lenexa, KS) media at 37 °C, unless indicated otherwise. BHS primary lung (BHSPL) cells were prepared from the lung tissue of a 2–3 month old fetus, as described previously (Shanthalingam et al., 2010). Human embryonic kidney 293T cells (ATCC Manassas, VA), along with BHSPL, were used for in vitro expression of the *lkt-plpE* construct. Bovine lymphoma (BL3) cells were used in cytotoxicity inhibition assays. 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; ThermoFisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA), while BL3 cells were cultured in Roswell Park Memorial Institute (RPMI; ThermoFisher Scientific, Waltham, MA) medium supplemented with 10% fetal bovine serum, unless indicated otherwise. BHSPL cells were cultured in DMEM supplemented with 15% fetal bovine serum.

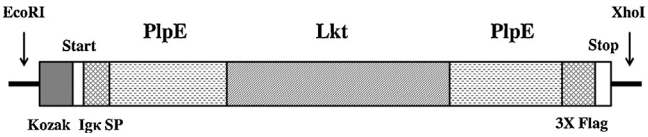

Fig. 1. Schematic representation of the *lkt-plpE* synthetic chimeric gene. The synthetic gene contained the *lkt* domain flanked by two *plpE* domains. The immunoglobulin κ (Igκ) signal peptide and a C-terminal 3X flag tag were incorporated for efficient secretion and detection, respectively. The EcoRI and XhoI restriction sites were introduced to facilitate subsequent cloning into the expression vector.

2.2. Construction of lkt-plpE chimeric gene

One Lkt neutralizing epitope has been mapped to lie between amino acid residues 841 and 872 (Lainson et al., 1996). The *lkt* gene in *M. haemolytica* strain WSU-1 was PCR-amplified and sequenced. An Lkt fragment comprising amino acids 292-954, from the WSU-1 strain was selected for the construction of an *lkt-plpE* chimeric gene. The surface-exposed immunogenic domain of outer membrane protein PlpE is comprised of the characteristic imperfect hydrophilic hexapeptide repeats (Ayalew et al., 2004). The PlpE immunogenic domain spanning amino acids 46-172 (GenBank: AAC82640.1), containing the hexapeptide repeats was selected for the chimera. A chimeric *lkt-plpE* gene encoding one Lkt-neutralizing domain flanked by two immunogenic PlpE domains (Fig. 1) was submitted to Eurofins (Dayton, OH) for codon optimization and synthesis. To this chimeric gene, an N terminal Igκ signal sequence (GenBank: AAA38778.1) and a C terminal 3xFLAG (Sigma, St. Louis, MO) epitope-coding sequence were added, to ensure efficient secretion and detection, respectively. The chimeric gene was flanked by EcoRI and XhoI restriction sites. Expression of the chimeric gene was under the regulation of the cytomegalovirus immediate early promoter. The presence of O-linked and N-linked glycosylation sites in the chimeric protein were predicted using the NetOGlyc and NetNGlyc servers (Center for Biological Sequence Analysis at the Technical University of Denmark, Kongens Lyngby, Denmark). The hydrophilicity of the chimeric protein was estimated from the GRAVY (grand average of hydropathy) value, calculated by adding the hydropathy value for each amino acid residue and dividing by the length of the sequence.

2.3. Construction of pENTR-Sai recombinant plasmid

A KpnI/HindIII fragment (derived from the expression plasmid pEGFP-C1) containing the enhanced green fluorescence protein (*eGFP*) gene cassette was kindly provided by Dr. Shafiqul Chowdhury (Louisiana State University, Baton Rouge, LA). A multiple cloning site (MCS) containing the restriction sites for enzymes BamHI, HindIII, KpnI and SphI (in sequence) was TOPO^{R}-cloned into the commercial vector pENTR^{TM}/D-TOPO^{R} (ThermoFisher Scientific, Waltham, MA), according to the manufacturer's instructions, generating the cloning vector pENTR-MCS. The KpnI/HindIII *eGFP* fragment was then inserted into the cloning vector pENTR-MCS, generating the expression vector pENTR-GFP. The NheI site within the e*GFP* fragment was replaced with an EcoRI site in this vector by PCR, allowing for *eGFP* ORF excision using the enzymes EcoRI and XhoI. The synthetic chimeric *lkt-plpE* gene was thus inserted in place of the *eGFP* ORF, generating the expression vector pENTR-Sai.