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Immunization of bighorn sheep against *Mannheimia haemolytica* with a bovine herpesvirus 1-vectored vaccine

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ABSTRACT

Mannheimia haemolytica is an important pathogen of pneumonia in bighorn sheep (BHS), consistently causing 100% mortality under experimental conditions. Leukotoxin is the critical virulence factor of *M. haemolytica*. In a 'proof of concept' study, a vaccine containing leukotoxin and surface antigens of *M. haemolytica* induced 100% protection in BHS, but required multiple booster doses. Vaccination of wildlife is difficult. BHS, however, can be vaccinated at the time of transplantation, but administration of booster doses is impossible. A vaccine that does not require booster doses, therefore, is ideal for vaccination of BHS. Herpesviruses are ideal vectors for development of such a vaccine because of their ability to undergo latency with subsequent reactivation which obviates the need for booster administration. The objective of this study was to evaluate the potential of bovine herpesvirus 1 (BHV-1) as a vector encoding *M. haemolytica* immunogens. As the first step towards this goal, the permissiveness of BHS for BHV-1 infection was determined. BHS inoculated with wild-type BHV-1 shed the virus following infection. The lytic phase of infection was superseded by latency, and treatment of latently-infected BHS with dexamethasone reactivated the virus. A recombinant BHV-1-vectored vaccine encoding a leukotoxin-neutralizing epitope and an immuno-dominant epitope of the outer membrane protein PlpE was developed by replacing the viral *glycoprotein C* gene with a *leukotoxin-plpE* chimeric gene. Four of six BHS vaccinated with the recombinant virus developed significant leukotoxin-neutralizing antibodies at day 21 post-vaccination, while two of six BHS developed significant surface antigen antibodies at day 17 post-vaccination. These antibodies, however, were inadequate for protection of BHS against *M. haemolytica* challenge. These data indicate that BHV-1 is a suitable vector for immunization of BHS, but additional experimentation with the chimeric insert is necessary for development of a more efficacious vaccine.

1. Introduction

The population of bighorn sheep (BHS) in North America has drastically declined from an estimated two million animals in the 1800s to less than 70,000 at the present time [1]. Pneumonia is the primary disease responsible for this drastic decline. Pneumonia outbreaks in wild BHS herds result in all age mortality as high as 90% [2]. Following recovery, the herd experiences pneumonia induced lamb mortality for several years [3–5]. Hence, a pneumonia outbreak engenders a diminishing herd population. Current management strategies to replenish affected herds involve transplantation of BHS from healthy herds. Despite extensive transplantation efforts over the decades, the problem of BHS pneumonia still remains. Therefore, there is an urgent need for additional disease management strategies.

Mannheimia haemolytica is an important pathogen of pneumonia in BHS. It is frequently detected in the lungs of pneumonic BHS [6]. Under experimental conditions, this bacterium consistently causes 100% mortality in BHS [7–9]. *M. haemolytica* possesses several virulence factors including the capsule, outer membrane proteins (OMPs), lipopolysaccharide and leukotoxin (Lkt). Lkt-deletion mutants of *M. haemolytica* do not cause

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mortality in BHS [9], and therefore Lkt is recognized as the critical virulence factor in *M. haemolytica*- caused pneumonia in BHS.

Previously, in a 'proof of concept' study, we developed an experimental vaccine consisting of *M. haemolytica* Lkt and surface antigens [10]. The vaccine comprised of concentrated logarithmic-phase culture supernatant fluids obtained from *M. haemolytica* and *Biberstinia trehalosi* cultures. Although this vaccine induced 100% protection in captive BHS, it required multiple booster doses. 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 booster doses is available.

Herpesviruses are ideal vectors for development of such a vaccine. Their ability to undergo latency with subsequent reactivation obviates the need for booster doses since the heterologous antigens carried by the vector, would also be expressed during lytic infection following reactivation, when the animals come under stress. An additional advantage is their large genome which is amenable to insertion of heterologous genes without impairing viral replication. Bovine herpes virus-1 (BHV-1) is also a suitable vector for BHS on account of its low seroprevalence in this species [11].

Previously, we have shown that induction of antibodies to the Lkt-neutralizing epitopes and to the surface antigens of *M. haemolytica* confers protection of BHS, against virulent *M. haemolytica* challenge [10]. The outer membrane phospho-lipoprotein PlpE, is surface- exposed and present in all serotypes of *M. haemolytica* [12,13]. 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 [14]. Moreover, a chimeric Lkt-PlpE protein subunit vaccine containing Lkt-neutralizing and PlpE immunodominant epitopes conferred enhanced resistance to cattle from *M. haemolytica* challenge [15].

In the current study, we developed and evaluated a BHV-1-vectored vaccine carrying a chimeric *lkt-plpE* gene, encoding Lkt-neutralizing epitopes of *M. haemolytica* Lkt and the immunodominant surface-exposed region of *M. haemolytica* PlpE.

2. Materials and methods

2.1. Virus, Bacteria, and cells

The wild-type BHV-1 Cooper strain was used to construct the virus-vectored vaccine. Madin-Darby bovine kidney (MDBK) cells (ATCC, Manassas, VA) were used to propagate the virus. The *M. haemolytica* serotype A2 strain WSU-1, isolated from the nasopharynx of a domestic sheep [8], was used in the challenge experiments. Bacteria were grown in brain heart infusion (BHI; Remel, Lenexa, KS) media at 37 °C, unless indicated otherwise. Bovine lymphoma (BL3) cells (ATCC, Manassas, VA) were used in cytotoxicity assays to determine Lkt-neutralizing antibody titers. MDBK 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.

2.2. Construction of pENTR-GFP-gC and pENTR-Sai-gC recombinant plasmids

Expression plasmids pENTR-GFP and pENTR-Sai [16] were used to generate the recombination-compatible pENTR-GFP-gC and pENTR-Sai-gC plasmids. BHV-1 *glycoprotein C (gC)* downstream and upstream flanking regions were amplified using the primers gCDP1 BamHI: 5′-ACGGATCCAGTGCCGCTTCGCTCACTTTACG-3′; gCDP2 HindIII: 5′-GCCAAGCTTCCAAAGACCACGCATGTACAATTTC-3′ and gCUP1 KpnI: 5′-CGGGTACCTTTATTCGTGGTCCGGGATTGAG CAC-3′; gCUP2 SphI: 5′-AGCTGCATGCGCCACGTTGCGTTATTTGGG-3′, respectively (Fig. 2A). To generate the homologous recombination (HR)-compatible pENTR-GFP-gC vector, *gC* upstream and downstream flanking regions were inserted into pENTR-GFP vector via the unique BamHI, HindIII, KpnI and SphI restriction sites. Similarly, to generate the HR-compatible pENTR-Sai-gC vector, the *gC* downstream flanking region was inserted into pENTR-Sai via the KpnI and SphI restriction sites. Both pENTR-GFP-gC and pENTR-Sai-gC plasmids were linearized prior to co-electroporation with viral DNA.

2.3. Generation and characterization of the recombinant BHV-1 vectored vaccine

Infectious BHV-1 DNA was prepared from virus-infected cells as described previously [17], with minor modifications of the protocol. Briefly, the virus was purified from cell debris, using sucrose cushion centrifugation (25,000 x rpm for 2.5 h at 4 °C). Subsequently, the purified virus particles were lysed by the addition of SDS (2% w/v) and proteinase K (1.8 mg/ml). The viral DNA was separated from proteins (using buffer-saturated phenol and 24:1 chloroform:isoamylalcohol); precipitated (using sodium acetate and absolute ethanol) and cleansed (using 80% ethanol) for use in transfections. Freshly cultured MDBK cells (1×10^7) were co-electroporated (0.27 kV; 0.95 F) with infectious viral DNA and linearized pENTR-GFP-gC plasmid, at a 1:9 (viral DNA: plasmid DNA) ratio. Immediately after electroporation, MDBK cells were cultured in DMEM supplemented with 15% fetal bovine serum. On appearance of infected cells/plaques (usually in 2–5 days post electroporation), the infected cells/plaques were screened for the presence GFP and positive plaques were picked. The picked plaques were subjected to five rounds of plaque-purification, to remove any non-fluorescing (non-recombinant) plaques, prior to expansion *in vitro*. To construct the recombinant BHV-1-vectored vaccine carrying the *lkt-plpE* chimera gene (BHV-1 Δ gC-Lkt-PlpE), another HR step mediated by co-electroporation of MDBK cells with infectious recombinant BHV-1 Δ gC-GFP viral DNA and linearized pENTR-Sai-gC plasmid, was similarly accomplished. Successful insertion of the chimeric gene in place of *GFP* in the viral DNA was preliminarily determined on the basis of absence of fluorescence. To confirm insertion, an 'out-out' PCR using primers gC-F: 5′-GACGACTAC GAAAACTACGACGAG-3′ and gC-R: 5′-TAATGGCGCACGTGGG CATCTG-3′ was performed. These primers amplify the entire *gC*, *GFP* or *lkt-plpE* gene in the wild-type, BHV-1 Δ gC-GFP or BHV-1 Δ gC-Lkt-PlpE viruses, respectively. The supernatant fluid obtained from MDBK cells infected with the BHV-1 Δ gC-Lkt-PlpE vaccine virus was probed for the presence of the chimeric protein by western blot analysis using antibodies specific to the FLAG epitope as described previously [16].

2.4. Inoculation of BHS with wild-type BHV-1

Prior to developing the BHV-1-vectored vaccine, a pilot study was conducted to confirm the permissiveness of BHS for BHV-1 infection. One adult female BHS aged 2 years was intra-nasally inoculated with 4×10^7 PFU of wild-type BHV-1 on day 0, using an atomizer (Develbiss Healthcare, Somerset, PA). Another sentinel adult (male) BHS was co-housed with this ewe to detect virus transmission. Prior to the inoculation, the animals were swabbed (nasally) and bled to ensure that they were culture- and sero-negative for BHV-1. Following inoculation, nasal swabs (in 1 ml

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