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Immunity of cattle following vaccination with a *Mannheimia haemolytica* chimeric PlpE–LKT (SAC89) protein

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ABSTRACT

We developed several chimeric PlpE–leukotoxin (LKT) constructs containing the major epitope of *Mannheimia haemolytica* outer membrane lipoprotein PlpE (epitope R2) and the neutralizing epitope of *M. haemolytica* LKT (NLKT) [Ayalew et al. *Mannheimia haemolytica* chimeric protein vaccine composed of the major surface-exposed epitope of outer membrane lipoprotein PlpE and the neutralizing epitope of leukotoxin. Vaccine 2008;26(38):4955–61]. Vaccination of mice with these PlpE–LKT chimeric proteins stimulated anti-PlpE antibodies that caused complement-mediated bacteriolysis of *M. haemolytica* as well as neutralizing anti-LKT antibodies. Chimeric protein SAC89, which contains two copies of R2 and two copies of NLKT, generally stimulated the best overall responses in mice. The objectives of the current study were: (1) to determine through a dose titration study if vaccination of cattle with SAC89 stimulated antibodies to both PlpE and LKT and (2) evaluate SAC89-induced immunity against experimental *M. haemolytica* challenge of cattle. In the dose titration study, vaccine doses ranged from 100 to 400 μg. SAC89 significant anti-*M. haemolytica* surface and LKT antibodies were detected following vaccination with each dose. The vaccination/challenge study was conducted with 30 weaned beef cattle distributed among four groups: Control (no vaccine), 100 μg SAC89, *M. haemolytica* Bacterin, and SAC89 + *M. haemolytica* bacterin. On day 42 after two vaccinations, cattle were challenged transthoracically with *M. haemolytica*. There was significant reduction ($p < 0.05$) in lesion scores for the SAC89 + bacterin-vaccinated group (74.6% reduction compared to control lesion scores) when compared to the other groups (34.7% and 35.6% reduction compared to control lesion scores). Evaluation of antibody responses demonstrated that the control group failed to develop antibody responses to *M. haemolytica* surface antigens or to LKT. Bacterin-vaccinated cattle developed anti-*M. haemolytica* antibodies after the second vaccination. SAC89- and SAC89 + bacterin-vaccinated groups developed significant antibody responses 14 days after the first vaccination and further significant increases in antibodies after the second vaccination.
Conclusions: Vaccination with the chimeric protein SAC89 in conjunction with a *M. haemolytica* bacterin stimulated significant protection against a severe transthoracic challenge with the bacterium.

1. Introduction

The major cause of severe bacterial pneumonia in cattle is *Mannheimia haemolytica* serotype 1 (S1), and current vaccines against *M. haemolytica* are only moderately efficacious [1]. Shewen and Wilkie [2] demonstrated that immunity against *M. haemolytica* requires antibodies against leukotoxin (LKT), which causes necrosis, apoptosis or activation of ruminant leukocytes, as well as antibodies against bacterial cell surface antigens. The important surface immunogens needed to stimulate protective immunity against *M. haemolytica* are still being studied.

Several studies implicate outer membrane proteins (OMPs) as immunologically important surface antigens [3–11]. Our laboratory demonstrated that high antibody responses to a major 45-kDa outer membrane lipoprotein, PlpE, correlated with resistance against experimental challenge [9,11,12]. The *plpe* gene was cloned, sequenced, characterized, and expressed [12]. We also demonstrated that cattle vaccinated with commercial *M. haemolytica* vaccines to which 100 μg of recombinant *M. haemolytica* S1 PlpE (rPlpE) was added had significantly greater resistance against experimental challenge with either S1 or S6 than did cattle vaccinated with the commercial vaccine alone [4,5]. Epitope-mapping studies in our laboratory found that the major epitope region of *M. haemolytica* S1 PlpE, designated Region 2 (R2), consists of eight imperfect hexapeptide repeats of QAQNAP located near the N-terminal region, and antibodies to the R2 region stimulate complement-mediated bacteriolysis [12]. The R2 region of PlpE from S1 was nearly identical to the R2 region for S6 isolates [13].

LKT is a member of the RTX family of toxins and is encoded by the *lktA* gene in *M. haemolytica*. High LKT-neutralizing antibody titers

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correlate with resistance against *M. haemolytica* challenge, and the epitope involved in neutralization of LKT has been localized to a 32 amino acid region near the C-terminus [2,14,15]. While attempts to study the immunogenicity of this epitope as a LacZ fusion in rabbits were not successful [14], a larger portion in the C-terminus (amino acids 713–939) induced a strong neutralizing antibody response when administered as part of a *Bordetella bronchiseptica* fimbrial protein – LKT chimera [15].

We recently reported on the development of several PlpE–LKT chimeric constructs containing the R2 epitope of PlpE and the neutralizing epitope of LKT (NLKT) [16]. Vaccination of mice with these PlpE–LKT chimeric proteins stimulated anti-PlpE antibodies that caused complement-mediated bacteriolysis of *M. haemolytica* as well as neutralizing anti-LKT antibodies. One particular chimeric protein, SAC89, which contains two copies of R2 and two copies of NLKT, generally stimulated the best overall responses in mice. The objectives of this study were: (1) to determine if vaccination of cattle with various doses of SAC89 stimulated antibodies to both PlpE and LKT and (2) to evaluate SAC89 alone or SAC89 and *M. haemolytica* bacterin in enhancing resistance against experimental *M. haemolytica* challenge of cattle.

2. Materials and methods

2.1. Cattle

In the study, 59 weaned Angus crossbred beef calves were used and housed in the Wendal Wallace Bovine Research Park at Oklahoma State University. Cattle had been screened for anti-*M. haemolytica* whole cell antibodies and found to have antibody concentrations of <0.5 ng of antigen-specific IgG as determined by ELISA (see below), which was found previously to be a normal background concentration for calves susceptible to challenge with *M. haemolytica* S1 (A.W. Confer, unpublished data). Upon arrival at the research facility, all calves were vaccinated with a 7-way clostridial and leptospiral vaccine, and treated with an anthelmintic. The calves received free-choice native grass hay supplemented with grain ration throughout the study. All studies were approved by the Oklahoma State University Institutional Animal Care and Use Committee (protocol #182).

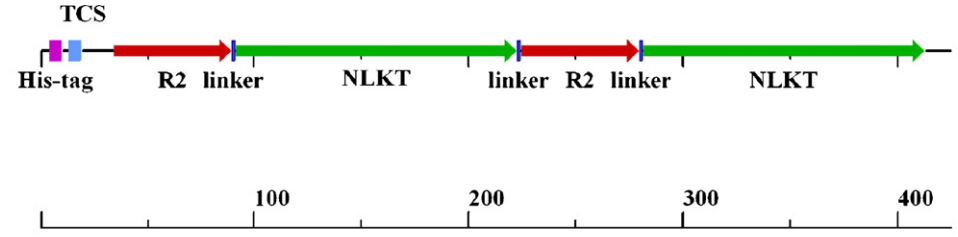
2.2. Enzyme-linked immunosorbent assay (ELISA)

Sera were assayed for anti-*M. haemolytica* IgG antibodies using ELISA against LKT, formalin-killed whole bacterial cells (WC), and SAC89 as previously described [3,4,17,18]. Ninety-six-well microtiter plates were coated with WC at a concentration equivalent to10^8 CFU/well, 50 ng/well each of LKT or SAC89 at 37 °C for 1–2 h or 4 °C overnight. After three washes each serum sample was diluted in phosphate-buffered saline (PBS)–Tween20 + 1% BSA and loaded onto the plate in triplicates at 100 μl per well. Serum dilutions for the various assays were 1:800 for WC, 1:1600 for LKT, or 1:400 for SAC89, which were in the linear range of established dilution curves. Plates were incubated on a rocking platform for 1 h at 37 °C and washed three times. Affinity-purified horseradish peroxidase-conjugated goat anti-bovine IgG [H+L] (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) diluted at 1:400 was used as secondary antibody and o-phenylenediamine (OPD) tablets, (Amresco, Inc., Solon, Ohio) were used as substrate. Color was developed in the dark and color intensity measured on Vmax kinetic Microplate reader at a 490 nm (Molecular Devices, Sunnyvale, CA). Antibody responses were expressed as nanograms of immunoglobulin binding based on a set of IgG standards on each plate.

2.3. Immunogens

The chimeric protein used in this study, SAC89 that comprises two copies of the immunodominant epitopes of PlpE (R2) and NLKT has a calculated molecular mass of 46,864.5 Daltons. The design, construction, expression and purification of SAC89 have been described by Ayalew et al. [16] (Fig. 1).

For bacterin preparation, *M. haemolytica* Oklahoma strain (a virulent S1 isolate) was plated on Brain Heart Infusion agar supplemented with 5% sheep blood and incubated at 37 °C and 5% CO_2 overnight. An inoculum of 5 ml was prepared by transferring a single colony into 5 ml of proprietary bacterial growth medium (Bacterin Growth Medium A [BMA], SolidTech Animal Health, Inc., Newcastle, OK) in 50 ml centrifuge tube and growing in a 37 °C shaker incubator (200 rpm) for 8 h. The entire growth (5 ml) was transferred to fresh 500 ml BMA and incubated overnight in a shaker incubator (180 rpm) at 37 °C. Bacterial cells were harvested at 6000 × *g*, and resuspended in PBS. The cells were washed three



His-tag: HHHHHH

TCS: Thrombin Cleavage Site (LVPRGS)

R2: TPNHPKPVLVPKTQNNLQAQNVPQAQNASQAQNAPQAQNAPQAQNAPQVENAPQA

NLKT: SDSNLKDLTFEKVKHNLVITNSKKEKVTIQNWFREADFAKEVPNYKATKDEKIEEIIGQN

GERITSKQVDDLIAKGNGKITQDELSKVVDNYELLKHSKNVTNSLDKLISSVSAFTSSND

SRNVLVAPTSM

Linker: RS

Fig. 1. Physical map of SAC89.