Ocular immune responses in steers following intranasal vaccination with recombinant *Moraxella bovis* cytotoxin adjuvanted with polyacrylic acid

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Running Head: Intranasal *M. bovis* cytotoxin subunit vaccine

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ABSTRACT. Infectious bovine keratoconjunctivitis (IBK) caused by *Moraxella bovis* (*M. bovis*) is the most common eye disease of cattle. The pathogenesis of *M. bovis* requires the expression of pili that enable the organism to attach to the ocular surface and an RTX (repeats in the structural toxin) toxin (cytotoxin; hemolysin) which is cytotoxic to corneal epithelial cells. In this pilot study ocular mucosal immune responses of steers were measured following intranasal (i.n.) vaccination with a recombinant *M. bovis* cytotoxin adjuvanted with polyacrylic acid. Beef steers were vaccinated with either 500 µg (n=3) or 200 µg (n=3) of recombinant *M. bovis* cytotoxin plus adjuvant. Control group steers (n=2) were vaccinated with adjuvant alone and all steers were booster on day 21. Antigen specific tear IgA and tear IgG, tear cytotoxin neutralizing antibody responses, and serum cytotoxin neutralizing antibody responses were determined in samples collected pre-vaccination and on days 14, 28, 42, and 55. Changes in tear antigen specific IgA levels from day 0 to days 28, 42, and 55 were significantly different between groups, however, in post hoc comparisons between individual group pairs at the tested time points, the differences were not significant. Results suggest that i.n. vaccination of cattle with recombinant *M. bovis* cytotoxin adjuvanted with polyacrylic acid effects changes in ocular antigen specific IgA concentrations. Use of intranasally administered recombinant *M. bovis* cytotoxin adjuvanted with polyacrylic acid could provide an alternative to parenteral vaccination of cattle for immunoprophylaxis against IBK.

KEYWORDS: *Moraxella bovis*; cytotoxin; intranasal; polyacrylic acid; IgA; vaccine
INTRODUCTION

Infectious bovine keratoconjunctivitis (IBK; “pinkeye”) is the most common eye disease of cattle and causes corneal ulceration, corneal edema, blepharospasm, photophobia, and lacrimation in affected animals; young animals are most often affected. The disease occurs most commonly in cattle populations during summer periods in association with risk factors such as ultraviolet radiation, dust, plant awns, and flies. In severe cases, rupture of the cornea results in permanent blindness. Along with economic losses associated with treatment and prevention of IBK, there are individual animal costs associated with reduced animal well-being, comfort, and welfare. The etiologic agent of IBK has long been considered to be Moraxella bovis (M. bovis) (1). In 2007 another Moraxella species, Moraxella bovoculi (M. bovoculi), was reported that had been isolated from calves with IBK in northern California (2). Challenge inoculation of calves with M. bovoculi has not supported a direct causal role for M. bovoculi in corneal ulceration associated with IBK (3), and at the present time, M. bovis remains the only organism for which Koch’s postulates have been established with respect to IBK (1).

The pathogenesis of M. bovis requires the expression of pilin for attachment to the corneal surface (4-6) and cytotoxin (hemolysin; cytolysin) that mediates damage to corneal epithelium leading to ulceration (7-9). Pilus based vaccines reduce the incidence and the severity of IBK (10-14), however, the presence of multiple pilus serogroups (15) coupled with the potential for pilin gene inversions (16) increases antigenic variability and may result in antigenic switching allowing M. bovis to evade a host immune response in animals vaccinated with pili (12). In contrast to pilus antigens, the M. bovis cytotoxin (MbxA) is more highly conserved amongst isolates (17). Cattle with IBK develop a systemic immune response to cytotoxin (18-21) and antihemolysin antibodies to one strain of M. bovis were shown to
neutralize hemolysins from different strains of *M. bovis* (20). Calves vaccinated with a partially purified cytotoxin were protected against IBK following challenge with a heterologous *M. bovis* strain (18) and protection against IBK was observed in calves vaccinated with a partially purified native *M. bovis* cytotoxin vaccine (22).

The efficacy of a subcutaneously administered recombinant *M. bovis* cytotoxin subunit vaccine against naturally occurring IBK was previously evaluated (23). While significantly reduced cumulative proportions of IBK-affected calves were found in vaccinates compared to control group calves during certain weeks of that trial, significant reductions in vaccinates were not maintained over the 20 week observation period spanning a typical pinkeye summer season. In subsequent studies to further refine the recombinant cytotoxin vaccine, additional antigens were added including conserved *M. bovis* pilin fragments (24) and recombinant *M. bovoculi* cytotoxin (25).

Most vaccine studies to prevent IBK have evaluated occurrence of IBK amongst vaccinates and controls that received a parenterally administered vaccine. Given the mucosal localization of IBK, it is rational to consider delivery of an *M. bovis* vaccine by a mucosal route. To date 4 studies have examined mucosal vaccination against IBK. Two of these studies evaluated an *M. bovis* bacterin administered by aerosol (26, 27), one evaluated a native *M. bovis* pilus antigen administered by the intranasal (i.n.) route (28), and one evaluated an *M. bovis* bacterin administered by the intraocular route (29). To the author’s knowledge, no published studies have evaluated ocular and systemic immune responses of cattle following i.n. vaccination with recombinant *M. bovis* cytotoxin. The following pilot study was done to determine whether i.n. administration of a recombinant *M. bovis* cytotoxin vaccine adjuvanted with a mucoadhesive
polymer (polyacrylic acid) could elicit ocular and systemic anti-cytotoxin antibody responses in beef steers.

MATERIALS AND METHODS

Animals. The experimental procedures for this study were approved by the University of California Institutional Animal Care and Use Committee (protocol #16585). Steers used for this study were maintained on a feedlot finishing ration throughout the study period at the University of California, Davis, Department of Animal Science campus feedlot. The study animals included 8 Angus and Angus-Hereford crossbred steers aged 336 to 380 days and weighing 380 to 539 kg. By the day of enrollment (Day 0), all steers had received both primary and booster vaccinations against respiratory viral pathogens (infectious bovine rhinotracheitis-parainfluenza 3- respiratory syncytial virus (INFORCE™ 3 (intrasal); Zoetis, New Jersey, USA), Clostridium spp. and Histophilus somni (ULTRABAC® 7/SOMUBAC®; Zoetis, New Jersey, USA), and Mannheimia haemolytica (ONE SHOT®; Zoetis, New Jersey, USA) and were dewormed with doramectin (DECTOMAX® POUR-ON; Zoetis, New Jersey, USA). All steers had received oral selenium bolus supplementation as calves prior to arrival at the feedlot (Se 365 bolus selenium supplement; Pacific Trace Minerals Inc, California, USA). Prior to the day of enrollment steers were restrained in a hydraulic squeeze chute and both eyes were examined; all enrolled steers were determined to have 2 clear corneas without evidence of corneal opacification suggestive of previous IBK. The 55 day trial began on September 28, 2011.

Antigen Preparation. Details regarding the molecular cloning and expression of the recombinant carboxy terminus (amino acids 590 through 927) of M. bovis cytotoxin (MxA) as inclusion bodies in E. coli have been previously described (23). Expressed inclusion bodies were
solubilized in buffer containing 4M urea, 0.25% Triton X-100, 5 mM Tris-HCl (pH 7.5) and 1 mM EDTA and then chromatographed (HiLoad™ 16/60 Superdex™200 prep grade column; Amersham Pharmacia Biotech Inc., New Jersey, USA) in buffer containing 8M urea, 200 mM NaCl, 1mM EDTA, 10 mM Tris-HCl; pH 7. Peak fractions were identified by SDS-PAGE, pooled and the recombinant protein was dialyzed against water at 4°C (Slide-A-Lyzer® Dialysis Cassette (Extra Strength) 10,000 MWCO; Thermo Fisher Scientific Inc., Illinois, USA) and then harvested. During dialysis the recombinant protein precipitated; the final recombinant protein was quantitated (BCA Kit; Thermo Fisher Scientific Inc., Illinois, USA) prior to vaccine formulation.

**Vaccine formulation.** The adjuvant (CARBIGEN™; MVP Technologies, Nebraska, USA) was added to the precipitated antigen to 9% v/v, the vaccine was mixed vigorously with shaking for 1 min at room temperature, and the final pH was adjusted to approximately 7 (pH 5-10 strips; EMD Chemicals Inc., New Jersey, USA) with 10 N NaOH. Following overnight storage at 4°C, vaccines were warmed to room temperature and the pH was readjusted to 7 if necessary. To reduce viscosity of the final vaccine, 9% NaCl was added to achieve a final salt concentration of approximately 0.1%. At this concentration the final vaccine could be easily expelled with a 3 cc syringe attached to a 14 cm catheter (Sovereign™ 3½ Fr Tom Cat Catheter, Tyco Healthcare Group LP, Massachusetts, USA). Vaccines were formulated to deliver 500 µg or 200 µg in a 2 ml volume. The control vaccine consisted of sterile water to which adjuvant was added as described above.

**Vaccination and sample collection.** The order in which the vaccines were administered to steers as they presented through the cattle chute on the day of vaccination (day 0) was predetermined by selecting each group assignment (3 for each of the 200 µg and 500 µg dose...
groups), and 2 controls from a hat without replacement. On day 0, the steers were administered
the 2 ml vaccine intranasally, 1 ml per nostril, with the heads restrained with nylon halters in an
elevated position; this head position was maintained for approximately 1-2 minutes following
administration to help ensure retention of the vaccine in the nasal cavity. Booster vaccinations
were administered similarly on day 21. Steers were housed in separate study groups throughout
the study.

Serum and tear samples were collected prior to vaccination on day 0 and on days 14, 28, 42, and 55. For sera, whole blood (10 ml) was collected by coccygeal venipuncture into serum separator tubes with no additive (Tyco Healthcare Group LP, Mansfield, MA, USA), allowed to clot and then centrifuged (2000 \times g) for 20 minutes, and then serum was harvested. Tears were collected from both eyes of each steer by placement of a cotton dental roll (Patterson Dental Supply, Inc, St. Paul, MN, USA) under the lower eyelid until the rolls were saturated. Once removed, rolls were processed and tear fluids were collected as previously described (23). Tears collected from both eyes were pooled together. Sera and tears were stored at -80°C until use.

The total protein concentration of collected tears was determined in triplicate (Pierce™ BCA Protein Assay Kit; Pierce Biotechnology, Illinois, USA).

**Tear and serum hemolysis neutralization assays.** A diafiltered retentate (DR) containing native *M. bovis* cytotoxin for use in tear and serum hemolysin neutralization assays was prepared as previously described (30) except that *M. bovis* was propagated in heart infusion broth (Bacto™ Heart Infusion Broth, Becton, Dickinson and Co., Maryland, USA) and the ultrafiltration/diafiltration of culture supernatant was performed using 2 cross-linked cellulose membrane cartridges in parallel according manufacturer directions (Vivaflow 200 Protein
Prior to use in hemolysis neutralization assays, tear and serum samples were heat inactivated at 56°C for 1 h. Neutralization assays were performed in 96 well cell culture plates (Nunclon F Delta Surface; Nunc A/S, Roskilde, Denmark). For serum neutralization assays, serial 2-fold dilutions of serum were made in TBS CaCl$_2$ buffer (50 mM Tris; 150 mM NaCl; 1.5 mM CaCl$_2$; pH 7.4); serum samples were assayed in duplicate. An equal volume of 1:32 diluted DR containing native *M. bovis* cytotoxin in chilled (4°C) TBS CaCl$_2$ buffer was added to the diluted serum and the plates were incubated on a rocker for 1 hour at 4°C. Next, an equal volume of a 1% suspension of washed packed bovine red blood cells (Defibrinated Bovine Blood, HemoStat Laboratories, California, USA) in TBS CaCl$_2$ buffer was added to the diluted serum and plates were incubated at a 45° angle in a humidified 37°C incubator for 3 hours. Plates were removed from the incubator and continued to incubate overnight at a 45° angle at room temperature to allow erythrocytes fully settle. Following overnight incubation plates were laid flat and individual wells were scored visually for the presence of hemolysis. The final serum cytotoxin neutralizing antibody titer was defined as the last dilution in which no hemolysis was observed. The geometric mean of 2 dilution endpoints was used as the final serum titer.

Tear neutralization assays were performed in 96 well plates in triplicate as described above for serum neutralization assays except that the DR containing native *M. bovis* cytotoxin was diluted 1:256 prior to adding it to the serially diluted tear samples; also, plates were incubated flat for 2 hours at 37°C after adding bovine erythrocytes. Following incubation, sedimented erythrocytes were gently resuspended by pipetting. Plates were then placed in a swinging bucket multiwell-plate carrier and centrifuged at 1280 × g for 10 min at 4°C.
Following centrifugation, 200 µl of supernatant from each well was transferred to a new 96 well plate and the optical density at 455 nm (OD$_{455}$) was measured in an ELISA plate reader (SpectraMax 250; Molecular Devices Corp., California, USA). The TBS CaCl$_2$ buffer alone and TBS CaCl$_2$ buffer with DR served as respective negative and positive (maximum lysis) controls (2 wells each) on each plate. To determine the final tear cytotoxin neutralizing antibody titer, the percent of maximum lysis was calculated by dividing the sample OD$_{455}$ by the average of the lysis positive control wells. The tear cytotoxin neutralizing antibody titer was defined as the last serial dilution for which the OD$_{455}$ remained <25% of the OD$_{455}$ of the mean of the 2 lysis positive control wells. The geometric mean of 3 dilution endpoints was used as the tear titer. To account for variations in tear titers caused by varying tear protein concentrations amongst samples, a tear neutralizing index (NI) was calculated as the natural logarithm of the final tear titer divided by the mg of protein present in the initial 1:2 dilution of the tear neutralization assay.

**Tear antigen-specific ELISA.** The concentrations of antigen-specific IgA and IgG in tears were determined by ELISA. Assays were performed in flat well 96 well plates (Immulon® 4HBX Ultra-high Binding Polystyrene Microtiter Plates; Thermo Scientific, New York, USA) at room temperature. Wells were coated for 1 hour on a platform shaker with either 100 µl of the recombinant carboxy terminus of *M. bovis* cytotoxin (5 µg/ml) diluted in coating buffer (0.05 M sodium carbonate; pH 9.6) (for antigen specific IgA or IgG) or affinity purified sheep anti-bovine IgA or IgG (Bethyl Laboratories, Texas, USA) diluted 1:100 in coating buffer (for IgA or IgG standard curve). After coating, plates were washed 5 times in ELISA buffer (50 mM Tris; 0.14 M NaCl; 0.05% Tween 20; pH 8.0) and 200 µl blocking buffer (ELISA buffer plus 2% fish gelatin; Sigma Life Science, Missouri, USA) was added. The plates were
incubated for 1 h and then washed 5 times and either 100 µl of bovine reference serum (Bethyl Laboratories, Inc., Texas, USA) appropriately diluted to generate a standard curve or diluted tear samples (1:80 dilution) were added to each well. Duplicate and triplicate wells were run for each respective standard curve value and unknown tear sample. A positive control tear IgA and tear IgG sample included on each plate was diluted tears from a study calf identified during ELISA optimization to have high antigen specific tear IgA or IgG. Plates were then incubated for 1 hr on a platform shaker, washed 5 times in ELISA buffer, and 100 µl of either sheep anti-bovine IgG (1:100,000 in ELISA buffer) conjugated to horseradish peroxidase (HRP) (Bethyl Laboratories, Inc.; Texas, USA) or sheep anti-bovine IgA-HRP (1:35,000 in ELISA buffer; Bethyl Laboratories, Inc., Texas, USA) was added and plates were incubated for 1 hr on a platform shaker. Following incubation, plates were washed 5 times and enzyme substrate (TMB peroxidase substrate ELISA; Moss, Inc., Maryland, USA) was added. Reactions were stopped with 100 µl of 0.1 N HCl and the OD at 450 nm was measured on an automated ELISA plate reader (SpectraMax 250; Molecular Devices Corp., California, USA). The final concentrations of antigen specific tear IgA or IgG were determined from the slopes of the standard curves generated by use of commercial ELISA analysis software (MasterPlex ReaderFit (Version 2.0.0.68); Hitachi Solutions America, Ltd., MiraiBio Group, California, USA). The calculated IgA or IgG concentrations represented the mean of triplicate samples. If the OD of a sample differed by > 10% of the mean of the triplicate samples, that value was excluded from the data analysis. To correct for interplate variation in the IgA or IgG values from the positive control wells, the calculated sample IgA or IgG was corrected by the formula: [(calculated sample IgA or IgG value) × (mean of all test plate positive control IgA or IgG values/positive control IgA or IgG value of the test plate)]. To account for variations in tear IgA and tear IgG concentrations...
caused by varying concentrations of tear protein, a tear IgA ratio and tear IgG ratio were calculated by dividing the natural logarithm of the final calculated antigen specific tear IgA or IgG concentration (in ng/ml) by the natural logarithm of the total tear total protein (µg/ml).

**Statistical Analysis.** Initial data diagnostics using the Shapiro-Wilk test revealed that the data were not normally distributed and thus non-parametric analyses were used. Analysis of variance of simple changes (for tear IgA and tear IgG ratios) and fold changes (for serum titer and tear NI) at time intervals from day 0 to day 14 (d0-d14), day 0 to day 28 (d0-d28), d0 to d42 (d0-d42), and day 0 to day 55 (d0-d55) were determined using the Friedman test. Post hoc analyses of differences between the 3 groups were determined using the Wilcoxon signed rank test with Bonferroni adjustment for multiple comparisons and the Dunnett’s multiple comparisons test for non-parametric or repeated measures of ANOVA. Where applicable, a commercial statistical software program was used for the analysis (SAS 9.3 Version, North Carolina, USA). In the overall analyses a $P < 0.05$ was considered significant; for the Bonferroni adjustment a $P < 0.0167$ (0.05/3 comparisons) was considered significant.

**RESULTS**

To determine if the i.n. vaccine administered to the steers in this study stimulated antibody responses in tears and serum of vaccinated steers, d0-d14, d0-d28, d0-d42, and d0-d55 changes in tear antigen specific IgA and IgG ratios and fold changes in tear neutralization index and serum neutralizing titers were measured. The changes in these immune response variables for the 500 µg dose, 200 µg dose, and control groups are shown in Figures 1 (tear IgA ratio), 2 (tear IgG ratio), 3 (tear NI), and 4 (serum neutralizing titer). The tear IgA ratios were significantly different amongst the 3 groups ($P = 0.0104$) at the 4 tested time intervals. While the mean
changes in tear IgA ratios were increased relative to the control group for d0-d28, d0-d42, and
d0-d55, post hoc comparisons between the individual treatment group pairs were not
significantly different ($P > 0.017$ for Bonferroni adjustment and for the Dunnett’s method’s
calculated values being less than the critical values) at these time intervals. No significant
differences were identified between groups for changes in tear IgG ratio, tear NI, or serum
neutralizing titers.

**DISCUSSION**

For this study measurements of tear antigen specific IgG ratio changes were included in order to
help interpret results of the tear neutralization assay. Because tear IgG is selectively transferred
from blood to tears in cattle (31), the ability of a collected tear sample to neutralize native *M.
bovis* cytotoxin in the tear neutralization assay cannot entirely be attributed to the presence of
antigen specific IgA. The results showed that mean fold changes in tear neutralization indices in
the vaccinates were higher than control group steers especially at d0-d28 and d0-d55, however,
the overall lack of significance in the measured differences between groups for tear IgG ratio and
tear neutralization index made it impossible to clearly attribute any changes in the tear NI to
either IgA or IgG.

For this study we used as a positive control on each tear IgA/IgG ELISA plate a sample
from one of the study animals that we identified during ELISA optimization to have high antigen
specific tear IgA or IgG. While it would have been more appropriate to use a control sample
from a non-study animal, such material was unavailable. The ELISA assays utilized IgA (or
IgG) standard curves within each assay to determine the antigen specific Ig concentration, an
approach that we had been previously used in another study (23). In the present study the positive control on each plate was used as an additional way to help control for interplate variation. In addition, in our analyses we evaluated changes from baseline Day 0 values amongst the three study groups. These methods seemed adequate to overcome the limitation of not having material from a non-study animal as a positive control.

In one of the high dose vaccine group steers the tear antigen specific IgG ratio was over 2x the tear IgG ratio of the other 2 steers in that group at the d0-d14 time interval. This same calf also had a 4 fold change in serum neutralization titer over this same time frame. We suspect that in this particular animal, the measured high change in tear IgG ratio occurred because of an increase in serum IgG titer to \textit{M. bovis} cytotoxin. While the steers in this study were initially screened for the presence of normally appearing corneas and showed no evidence of clinical IBK throughout the trial, ocular cultures were not collected to determine if animals were infected with \textit{M. bovis} prior to enrollment. Previously, cattle without clinical IBK were shown to develop serum hemolysin (cytotoxin) neutralizing titers in the absence of clinical disease (20). If seroconversion without clinical evidence of IBK had occurred, it follows that for this particular study animal there was not a marked change in the tear IgA ratio from D0-D14. Previously it was shown that lacrimal IgA responses against \textit{M. bovis} antigens were associated with more severe cases of IBK (32).

Over many years of investigation into the pathogenesis and prevention of IBK, there have been varying conclusions as to the importance of local ocular immunity in determining resistance to IBK. In early studies it was observed that lacrimal secretions of calves with more severe IBK developed increased lacrimal fluid IgA against crude \textit{M. bovis} antigens and the presence of these antibodies seemed to reduce disease susceptibility following reinfection (32, 33). While authors
suggested that local ocular vaccination could be beneficial (32), a subsequent investigation that
evaluated immune responses in ocular fluids of subconjunctivally vaccinated vs. unvaccinated
calves concluded that ocular antibodies to *M. bovis* were not protective because clinical IBK
developed even in the presence of high tear hemagglutinating (HA) titers (34). An important
observation in that study, however, was that the percent of calves (both vaccinated and
unvaccinated) from which *M. bovis* could be isolated decreased as the mean tear HA titer
increased (34). Authors of a subsequent study that measured lacrimal fluid IgA and IgG using an
indirect fluorescent antibody test also reported that lacrimal antibodies did not prevent IBK (35).

In later studies, however, positive correlations were reported between specific antibodies
in lacrimal secretions as assessed by a passive hemagglutination test using tannic acid treated
sheep red blood cells sensitized with a whole *M. bovis* cell sonicate, amelioration of clinical IBK,
and declining numbers of *M. bovis* that could be isolated from conjunctival swabs of
experimentally infected calves (36). When an ELISA using a whole *M. bovis* cell antigen was
used to measure anti-*M. bovis* responses in tears of calves with experimentally-induced IBK, a
predominant IgA response to *M. bovis* was detected (37). When a whole *M. bovis* antigen
ELISA was used to quantitate humoral IgG and lacrimal IgA in calves with experimentally
induced IBK, an association between the presence of ocular IgA against *M. bovis* antigens and
resistance to reinfection was found (38). The authors suggested that vaccination by a route that
would stimulate mucosal IgA against *M. bovis* would be important in developing an effective *M.
bovis* vaccine (38).

When subcutaneous versus subconjunctival routes of vaccination were evaluated in a
study of a pilus-based vaccine, subconjunctival vaccinates were found to have greater resistance
to challenge infection (39). In the most recent study of a subconjunctival route of vaccination
with an autogenous \textit{M. bovis} bacterin, no difference was found between subconjunctival versus subcutaneous vaccinates (40). In neither of these studies were lacrimal antibody responses against \textit{M. bovis} antigens measured and so it is unknown whether the subconjunctival route of injection successfully stimulated a specific ocular fluid antibody response against \textit{M. bovis} antigens.

Investigations into non-parenteral routes of vaccination of cattle against IBK have reported mixed results. An aerosolized fimbriated \textit{M. bovis} bacterin was reported to be effective at preventing naturally occurring IBK (26, 27). In those studies ocular antibody responses against \textit{M. bovis} antigens were not reported. Presumably the vaccine which was administered with an atomizer could have exposed antigen at inductive sites on ocular, nasal, and oropharyngeal mucosal surfaces. More recently, an investigation of ocular IgA antibody responses following i.n. vaccination with purified \textit{M. bovis} pili plus various adjuvants reported that pili adjuvanted with QuilA or Marcol Span induced significant increases in anti-pili ocular IgA responses compared to adjuvant control calves (28). In that study, however, the anti-pili ocular IgA responses did not correlate well with either protection against \textit{M. bovis} infection or the development of IBK (28). Other studies previously demonstrated success of subcutaneously administered pilus-based vaccines at protecting calves against homologous but not heterologous challenge with \textit{M. bovis} of different pilus serogroups (14) and it is possible that heterologous pilus strains were circulating in the study herd in the report by Zbrun \textit{et al} which may have contributed to the development of IBK amongst vaccinates despite high tear anti-pilus IgA concentrations. In another recent study, calves were challenged 45 days following topical ocular vaccination with a killed \textit{M. bovis} bacterin alone or with recombinant human interleukin 2 and interferon \(\alpha\) (29). In that study ocular antibody responses were not reported, however, calves...
that received the *M. bovis* bacterin plus cytokines had the lowest proportion of IBK and lowest clinical IBK score following challenge (29).

In the present study a variety of different factors may have contributed to suboptimal performance of the experimental *M. bovis* subunit vaccine including adjuvant, antigen formulation, and method of delivery. For this study we chose to use a polymer of acrylic acid (PAA) as adjuvant and while it is possible that the use of a different adjuvant may have improved our success at stimulating an ocular anti-cytotoxin IgA response, PAA was a logical choice because of its demonstrated adjuvant properties (41), mucoadhesive qualities, and commercial availability. The antigen used for this study precipitated during the final steps of dialysis against water and it is possible that reformulation of this antigen in a soluble form may improve its antigenicity. For this study we delivered the vaccine through a 14 cm polypropylene catheter. Delivery of antigen to inductive lymphoid sites within the bovine nasal cavity could have been improved by use of a longer catheter capable of delivering vaccine to nasal-associated lymphoid tissues of Waldeyer’s ring including pharyngeal and tubal tonsils. Commercial i.n. vaccines that are currently marketed for use in cattle in the USA use a relatively short cannula for administration, however, these vaccines contain modified live agents that are capable of inducing immunity by virtue of localized infection, a benefit that a nasally-delivered recombinant protein subunit such as the antigen used in this study does not have.

In a previous study of subcutaneously-administered recombinant *M. bovis* cytotoxin we observed significantly higher antigen specific IgG in sera and tears as well as the lowest cumulative proportion of IBK in vaccinated versus control group calves (23). In that study we also observed the largest corneal ulcers in vaccinated calves, an observation that suggested that perhaps high levels of IgG in serum/tears could have contributed to increased ocular pathology.
due to complement fixation and attraction of neutrophils and subsequent release of degradative enzymes into the corneal stroma. The possibility for immune mediated damage in the eye associated with *M. bovis* infections provides a rationale for trying to augment ocular antigen-specific IgA responses by mucosal vaccination. Shallower and larger (in surface area) ulcers were reported in one study of calves that were immunosuppressed by treatment with hydroxyurea versus control calves 8 days following an *M. bovis* challenge (42). A similar study of normal versus immunosuppressed calves with differing concentrations of anti-*M. bovis* IgG in ocular secretions could provide further insight into the possibility of immune-mediated ocular injury during *M. bovis* infection.

In summary, the results of this study demonstrated that i.n. vaccination of healthy beef cattle with the carboxy terminus of recombinant *M. bovis* cytotoxin adjuvanted with the mucoadhesive polymer polyacrylic acid can stimulate changes in antigen specific ocular mucosal IgA. It is likely that the small sample size and high individual animal variation in measured immune response variables accounted for the lack of statistical significance in post-hoc testing between treatment groups. While the significance of these results are limited, the use of i.n. vaccination to increase anti-cytotoxin specific IgA in tear secretions of steers vaccinated with an *M. bovis* cytotoxin subunit antigen adjuvanted with polyacrylic acid appears promising. Further studies are underway to investigate whether refinements to this vaccine can improve ocular mucosal responses to *M. bovis* cytotoxin in cattle. Subsequent trials will be necessary to determine whether such responses can prevent naturally occurring IBK.

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FIGURE LEGENDS.

FIG 1 Individual and group mean (+ SE) values for changes in tear antigen specific tear IgA ratios from day 0 to day 14 (D0-D14), day 0 to day 28 (D0-D28), day 0 to day 42 (D0-D42), and from day 0 to day 55 (D0-D55) in steers that received primary and day 21 booster i.n. vaccination with 0 µg (control group; n=2; black circles/white rectangles), 200 µg (n=3; black squares/hatched rectangles), or 500 µg (n=3; black triangles/gray rectangles) recombinant *Moraxella bovis* cytotoxin adjuvanted with polyacrylic acid. The tear IgA ratios were significantly different amongst the 3 groups (P = 0.0104) at the 4 tested time intervals. Post hoc comparisons between the individual treatment group pairs were not significantly different (P > 0.017).

FIG 2 Individual and group mean (+ SE) values for changes in tear antigen specific IgG ratios from day 0 to day 14 (D0-D14), day 0 to day 28 (D0-D28), day 0 to day 42 (D0-D42), and from day 0 to day 55 (D0-D55) in steers that received primary and day 21 booster i.n. vaccination with 0 µg (control group; n=2; black circles/white rectangles), 200 µg (n=3; black squares/hatched rectangles), or 500 µg (n=3; black triangles/gray rectangles) recombinant *Moraxella bovis* cytotoxin adjuvanted with polyacrylic acid.
FIG 3 Individual and group mean (+ SE) fold changes in *Moraxella bovis* cytotoxin neutralization indices of tear samples from day 0 to day 14 (D0-D14), day 0 to day 28 (D0-D28), day 0 to day 42 (D0-D42), and from day 0 to day 55 (D0-D55) in steers that received primary and day 21 booster i.n. vaccination with 0 µg (control group; n=2; black circles/white rectangles), 200 µg (n=3; black squares/hatched rectangles), or 500 µg (n=3; black triangles/gray rectangles) recombinant *M. bovis* cytotoxin adjuvanted with polyacrylic acid.

FIG 4 Individual and group mean (+ SE) fold changes in serum neutralizing antibody titers to *Moraxella bovis* cytotoxin from day 0 to day 14 (D0-D14), day 0 to day 28 (D0-D28), day 0 to day 42 (D0-D42), and from day 0 to day 55 (D0-D55) in steers that received primary and day 21 booster i.n. vaccination with 0 µg (control group; n=2; black circles/white rectangles), 200 µg (n=3; black squares/hatched rectangles), or 500 µg (n=3; black triangles/gray rectangles) recombinant *M. bovis* cytotoxin adjuvanted with polyacrylic acid.
FIGURE 4