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Randomized controlled field trial to assess the efficacy of an intranasal *Moraxella bovis* cytotoxin vaccine against naturally occurring infectious bovine keratoconjunctivitis

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ABSTRACT

Background: Infectious bovine keratoconjunctivitis (IBK; pinkeye) is generally considered to be caused by corneal infections with *Moraxella bovis*. Previous studies demonstrated that *M. bovis* cytotoxin-specific mucosal immune responses in the bovine eye can be stimulated by intranasal vaccination with a recombinant *M. bovis* cytotoxin subunit adjuvanted with polyacrylic acid.

Methods: A randomized controlled field trial (two-arm parallel design with blinding) was conducted in beef steers in Northern California to determine if this vaccine could prevent naturally occurring IBK and/or reduce morbidity rates associated with this disease. Beef steers were vaccinated intranasally on days 0 and 21 with either a recombinant *M. bovis* cytotoxin subunit adjuvanted with polyacrylic acid (Vaccine group) or adjuvant alone (Control group). Eye examinations were performed on all steers every 7 days for 16 weeks to document the occurrence of IBK and to determine sizes of corneal ulcers. Serum and tear samples were collected on days 0, 42, and 112 from a subset of animals to measure changes in systemic and ocular immune responses to *M. bovis* cytotoxin.

Results: The cumulative proportion of steers that developed IBK after 16 weeks did not differ between groups. Variables related to disease severity were numerically lower in steers that received the experimental vaccine. IBK-affected Vaccine group steers had a significantly lower number of observation weeks with severe ulcers versus Control group steers. Cytotoxin-specific tear IgA was significantly higher in Vaccine group compared to Control group steers on day 112. *Conclusion:* Although the proportion of animals that developed corneal ulcers associated with IBK did not differ between groups, the lowered metrics of disease severity in vaccinated steers suggests that intranasal vaccination with recombinant *M. bovis* cytotoxin can reduce the severity of IBK in cattle.

1. Introduction

Infectious bovine keratoconjunctivitis (IBK; pinkeye) is the most common eye disease of cattle. For many years, an association between IBK and ocular infection with *Moraxella bovis* has been recognized [1]. The characterization of a newly identified *Moraxella* species, *Moraxella bovoculi*, from eyes of calves affected with IBK [2], suggests that there may be a role for other bacterial species in addition to *M. bovis* in the pathogenesis of IBK. While published challenge studies have not demonstrated a definitive role for *M. bovoculi* in the formation of corneal ulcers which are a clinical hallmark of IBK [3], data from diagnostic studies evaluating ocular flora in IBK-affected cattle documented the presence of *M. bovoculi* in a majority of cases of IBK [4,5]. It is possible that *M. bovoculi* functions as a risk factor for IBK as do agents such as *Mycoplasma* spp [5,6], infectious bovine rhinotracheitis virus (bovine herpesvirus) [7], insect vectors [8–10], plant awns, and solar irradiation [11].

Various *M. bovis* whole cell preparations or components have been tested as vaccine antigens to prevent IBK. Of the cellular components tested, most research has focused on parenterally administered pilin and cytotoxin. Pilin expression is necessary for *M. bovis* to attach to corneal epithelium [12–14]. Multiple pilus serogroups have been identified in *M. bovis* [15], and this variability in conjunction with a pilin gene inversion mechanism [16] is considered to contribute to the diversity between isolates that could enhance the ability of *M. bovis* to evade host immune responses [17].

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The *M. bovis* cytotoxin (hemolysin), MbxA [18], is an RTX toxin that is also necessary for pathogenesis [19–21]. MbxA is conserved among geographically diverse isolates [22] and IBK-affected cattle develop antihemolysin antibodies that cross-neutralize hemolysin from diverse strains of *M. bovis* [23]. These findings suggest that a vaccine antigen based on cytotoxin could be more widely effective as a vaccine antigen candidate versus pilin.

Multiple studies that have evaluated commercially available or autogenous vaccines against IBK have reported lack of efficacy of these parenterally administered vaccines [24–27]. An experimental parenteral vaccine that used a partially purified native *M. bovis* cytotoxin as antigen showed some efficacy against IBK [28]. Other parenterally-administered *M. bovis* cytotoxin subunit vaccines have also been investigated [29,30]. Results of these studies have been variable and further research is necessary to identify vaccine antigens, adjuvants, and/or routes of administration to improve vaccine technology in order to effectively prevent IBK.

Relatively little research has been published on mucosal application of *M. bovis* antigens and whether vaccines administered by either topical ocular or intranasal routes of administration can prevent IBK. Two studies reported that the administration of an *M. bovis* bacterin via aerosol was effective against IBK [31,32]. Intranasal vaccination with *M. bovis* pili in calves resulted in significantly increased anti-pilin IgA in tears; however, this response did not correlate with protection against IBK [33]. Reduction in overall IBK and disease severity were reported in experimentally infected calves that had previously been vaccinated by an ocular spray containing an *M. bovis* bacterin plus IL-2 and IFN-α [34].

To stimulate local ocular immunity against *M. bovis* cytotoxin, we previously evaluated a intranasally administered *M. bovis* cytotoxin subunit adjuvanted with polyacrylic acid [35,36]. These studies showed that this antigen-adjuvant combination stimulated ocular *M. bovis* cytotoxin antigen-specific responses in tear fluid. In the study reported here, we sought to examine the efficacy of an intranasally administered recombinant *M. bovis* cytotoxin subunit adjuvanted with polyacrylic acid against naturally occurring IBK in a randomized controlled field trial.

2. Methods

2.1. Study site and animals

This study was conducted during late spring and summer 2016 at the Sierra Foothills Research and Extension Center (SFREC), Browns Valley, CA. The SFREC has native oak woodland and annual grass pastures typical of beef cattle grazing areas in the Sierra foothills of Northern California. Beef cattle housed at this facility typically experience naturally occurring IBK during summer months. The study population was comprised of pure and crossbred weaned beef steers approximately 7–10 months of age that had not previously been vaccinated against *M. bovis* or *M. bovoculi*. The month prior to enrollment, steers received vaccinations against clostridial species (*Clostridium chauvoei*, *C. septicum*, *C. haemolyticum*, *C. novyi*, *C. sordellii*, *C. perfringens* types C and D), respiratory pathogens (bovine rhinotracheitis virus, bovine parainfluenza-3, bovine respiratory syncytial virus, and bovine virus diarrhea virus), *Anaplasma marginale*, and received antiparasitic treatment with moxidectin. Antiparasitic treatment was repeated again on study day 56. The study commenced on April 6 (study day 0; D0) and ended on study day 112 (July 27; D112) and was approved by the UC Davis Institutional Animal Care and Use Committee (protocol #18607).

2.2. Sample size, blinding, and randomization

A minimum sample size for the two study groups in this field trial was calculated using standard methods [37]. The proportions of vaccinated animals that developed IBK by 8 weeks following vaccination in a previous IBK vaccine study [38] were 23% and 28% for experimental vaccine and control groups, respectively. For the vaccine in this study to be considered effective for the prevention of IBK, the experimental vaccine would have to reduce the risk of developing IBK by at least 5%. Therefore, using a power of 80% and alpha = 0.05, it was determined that a minimum of 76 animals in each group would be necessary for this study.

Following preparation of the control and experimental vaccines used in this study (see below), vaccine bottles were designated as either 'A' or 'B' such that investigators were unaware of which vaccine product each steer was administered at primary (D0) and booster (D21) vaccinations and throughout the 112 day trial. To maintain approximately equal numbers of animals across the two study groups, a list of numbers was generated as 98 sets of 2 unique even or odd numbers (1 or 2) (https://www.randomizer.org/). Animals that were free of active IBK (see below) were vaccinated with either vaccine A (even number) or B (odd number) in the order that they entered the cattle chute.

2.3. Antigen production and vaccine formulation

A recombinant carboxy terminus subunit (amino acids 590 through 927) of the *M. bovis* cytotoxin (recombinant MbxA) was used as the experimental vaccine antigen. The protein subunit was expressed as inclusion bodies in *Escherichia coli* as previously described [30] and purified following previously described methods [35]. The final purified protein was quantitated (Pierce BCA Protein Assay; Thermo Fisher Scientific, Inc., Illinois). The relative abundance of recombinant MbxA in the final purified protein preparation that had precipitated following water dialysis was estimated by SDS-PAGE and Coomassie staining followed by gel analysis (NIH ImageJ; https://imagej.nih.gov/ij/). The final experimental vaccine antigen was stored at −20 °C until vaccine formulation.

Recombinant MbxA was adjuvanted with 10% polyacrylic acid (Carbigen™; MVP Technologies, Nebraska) following previously described methods [36]. The final experimental vaccine was designed to provide 500 μg of recombinant MbxA in a 2 ml dose. The control vaccine contained 10% polyacrylic acid diluted in water.

2.4. Pre-enrollment exam, enrollment, and sample collection

Steers enrolled in this study were pure and mixed breed beef steers that were not previously vaccinated against IBK. On D0 (day of enrollment), steers were restrained in a cattle chute and both eyes were examined for evidence of active IBK using direct visual examination with a penlight for corneal edema from presumptive corneal ulceration along with tearing and/or blepharospasm. Only animals free of active IBK were enrolled. Weights of steers were obtained on D7 and on D112. Following examination and vaccination, steers were commingled and maintained as a single cohort during the 112-day study period.

Prior to vaccination, tear samples from left and right eyes were collected and pooled from both eyes of the first 26 steers that were eligible for enrollment, and jugular venous blood samples (for serum collection) were collected from the first 52 steers that were eligible for enrollment as previously described [30]. Tear and whole blood samples were collected again on D42 (3 weeks post booster) and D112. Tear samples were only collected from eyes without evidence of active IBK during collections on D42 and D112.

Tears were harvested from cotton swabs by centrifugation (2000*g*; Beckman SX4750 rotor) at 4 °C for 15 min, decanted, and stored at −80 °C until use. Prior to use in tear neutralization assays and ELISAs, tear samples were heat inactivated for 1 h at 56 °C and tear protein was quantified (Pierce BCA Protein Assay).

Collected blood was allowed to clot prior to centrifuging (1400*g*; Beckman SX4750 rotor) for 10 min at 25 °C. Serum was harvested and stored at −80 °C until use. Prior to use in serum neutralization assays and ELISAs, serum samples were heat inactivated at 56 °C for 1 h.

2.5. Vaccination and blinding

Vaccines were administered intranasally in one nostril using a 3 ml Luer lock syringe attached to a 15 cm flexible nasal cannula with 3 small openings on the end (Equine Nasal Applicator; Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, Connecticut). During vaccination, an effort was made to elevate the head while the cannula was directed dorsal-medially along the nasal septum. Booster vaccinations were administered on D21. Investigators were blinded as to the contents of the vaccines that were administered during the study (either experimental vaccine (Vaccine group) or control vaccine (Control group)).

2.6. Post-enrollment examinations

Every 7 days for 16 weeks, all steers were gathered and both eyes were evaluated while animals were restrained in a squeeze chute. All ocular examinations were performed by a veterinarian (JAA) with extensive clinical experience diagnosing IBK; this individual was also blinded as to the vaccine type that each steer received. Both eyes were examined for the presence of corneal edema suggestive of IBK. If corneal edema was present, the eye was stained with fluorescein to determine if a corneal ulcer was present. If fluorescein staining revealed the presence of a corneal ulcer, the ulcer was considered to be due to IBK unless the pattern of fluorescein staining was more typical of a mechanical scratch (a linear or stellate pattern of fluorescein staining) or presence of a plant awn (pattern of corneal opacity that was not centrally located on the cornea and that was continuous with the limbus). Mechanical or plant awn-induced corneal ulcers were not considered as IBK unless a corneal ulcer was present at the next weekly observation. In such cases, the first day of ulceration due to IBK was considered to be the subsequent observation day following the initial mechanical scratch or plant awn-induced corneal ulcer. If present at the time of initial observation, plant awns were removed from the eye using a hemostat.

Ulcerated eyes attributed to IBK were assigned a corneal ulcer score (CUS) using a previously established 4 point scoring system [29]: 0 (no ulcer); 1 (maximal ulcer diameter $\leq$5 mm); 2 (maximal ulcer diameter $>$5 mm); or 3 (perforated corneal ulcer as assessed by evidence of globe rupture or visible prolapse of the iris). Eyes that received a CUS 3 during a weekly visit were subsequently scored depending on the size of the area of fluorescein uptake using the scoring system described above. Eyes with ulcers attributed to IBK were digitally photographed with a ruler held next to the eye for measurement of the corneal ulcer surface area (see below).

2.7. Treatments

Steers assigned a CUS = 2 were treated with a single dose of oxytetracycline (20 mg/kg; subcutaneously in the neck area) on the first observation day that a corneal ulcer attributed to IBK was observed. At a subsequent weekly observation following oxytetracycline treatment, if the ulcer appeared clinically worse, florfenicol was administered (40 mg/kg subcutaneously in the neck area). Steers with ulcers were evaluated for evidence of severe ocular pain by observing for eyelid closure/blepharospasm during ocular examinations. Flunixin meglumine (1 mg/kg IV) was administered to steers that were assessed to have severe ocular pain.

2.8. Corneal ulcer surface area measurement

The area of corneal ulceration in IBK-affected eyes (surface area measurement; SAM) was determined from digital photographs as previously described [38]. The average of three tracings was used as the final SAM. New ulcers that were $<$0.008 cm^2 were not counted as IBK. If more than one area of ulceration on a cornea was present and $\geq$ 0.008 cm^2, the final SAM for that eye was considered to be the sum of the SAMs of the component ulcers. The cumulative corneal ulcer surface areas for each IBK-affected steer was calculated as the sum of weekly SAMs (left plus right eye if both eyes were affected) across all observation days.

2.9. Tear and serum cytotoxin neutralization assays

A diafiltered retentate (DR) containing *M. bovis* cytotoxin (hemolysin) was prepared by heavily streaking *M. bovis* onto 6% cow blood agar plates and incubating at 35 °C for about 20 h. Bacterial cells were then scraped from agar plates and resuspended in heart infusion broth (HIB; Bacto™ Heart Infusion Broth; Becton, Dickinson, and Co., Sparks, Maryland) containing 1.5 mM $CaCl_2$. Once bacterial cells were resuspended, 500 ml flasks of HIB with 1.5 mM $CaCl_2$ were inoculated with resuspended *M. bovis* and incubated at 35 °C with shaking at 200 rpm for 5–6 h. Following incubation, cultures were centrifuged (6000*g*; Sorvall GS-3 rotor) for 15 min at 4 °C. Culture supernatants were sterile filtered using prechilled 0.2 μm polyethersulfone filters (Nalgene Rapid-Flow Filter, Thermo Scientific). The resulting filtrate was concentrated approximately 20 fold (Vivaflow 200 protein concentrator HY [30,000 MWCO]; Sartorius Stedim North America, Inc., New York) and then diafiltered as previously described [18]. The supernatant was kept chilled (4 °C) throughout this procedure. The final DR was aliquoted and stored at −80 °C until used in serum and tear cytotoxin neutralization assays.

Serum cytotoxin neutralizing titers were determined in triplicate as previously described [35], except that following the overnight incubation, plates were scanned (Epson Expression 1600), and determination of the last dilution without evidence of hemolysis was made by evaluating scanned images. The inverse of this dilution was defined as the serum cytotoxin neutralizing titer, and the geometric mean of 3 dilution endpoints was the final serum cytotoxin neutralizing titer.

Tear cytotoxin (hemolysin) neutralization assays were performed as previously described [35], except that tear samples were assayed in duplicate, and prior to serial two-fold dilution of tears, tear samples in the first well were standardized to a protein concentration of 2,000 μg/ml (designated 1:1 dilution). The final tear cytotoxin neutralizing endpoint titer was defined as the inverse of the last dilution before the percent neutralization fell below 50%. If percent neutralization was $<$50% in the 1:1 dilution, the titer was designated as 0.5. The geometric mean of 2 dilution endpoints was taken as the final tear cytotoxin neutralizing titer.

2.10. ELISA procedures

Recombinant cytotoxin (MbxA)-specific serum IgG, tear IgG, and tear IgA were quantitated by ELISA from tear samples collected on D0, D42, and D112 following previously published procedures [36]. For the tear IgA and tear IgG ELISAs, tear samples were diluted in ELISA buffer (EB; Tris-buffered saline (TBS) $CaCl_2$ buffer (50 mM Tris, 150 mM NaCl, and 1.5 mM $CaCl_2$ [pH 7.4]) to a standard total protein concentration (2.1 mg/ml) before diluting 1:10 (for tear IgA) or 1:2 (for tear IgG) in EB. Serum samples were diluted 1:400 in EB. In the tear IgA assays, if results were below the range of the standard curve at the 1:10 dilution, samples from all 3 sampling dates were diluted 1:2 in EB before re-running the assay. The conjugates used for ELISAs were either sheep anti-bovine IgG conjugated to horseradish peroxidase (HRP) diluted 1:100,000 (for serum IgG) or 1:75,000 (for tear IgG) or sheep anti-bovine IgA-HRP diluted 1:35,000 (for tear IgA) (Bethyl Laboratories, Montgomery, Texas). Positive controls were included on each ELISA plate as previously described [36]. A previously described formula was applied to correct for inter-plate variation between ELISA plates [39].

2.11. Data analysis

The primary outcome variable was the cumulative proportion of steers that developed IBK at 16 weeks (D112). Secondary outcome variables that were compared between the two study groups were:

cumulative proportion of steers that developed IBK after 8 weeks; days to heal for the initial ulcer event; proportions of animals with IBK that required treatment with oxytetracycline, florfenicol, or flunixin meglumine; number of observation weeks with maximal CUS 0 or 1 versus 2 or 3; weight changes between D7 and D112; cumulative corneal ulcer surface areas of IBK-affected steers; serum and tear *M. bovis* cytotoxin (hemolysin) neutralizing antibody titers, serum and tear cytotoxin-specific IgG, and tear cytotoxin-specific IgA concentrations on D0, D42, and D112. The Shapiro-Wilk test was used to determine if data were distributed normally. Chi-square was used to evaluate differences between groups in proportions of steers that developed IBK or required treatment with antibiotics or flunixin meglumine, and differences between IBK-affected animals in numbers of observation weeks where the maximal CUS was 0 or 1 versus 2 or 3. An unpaired *t*-test was used to evaluate weight changes between D7 and D112. Intragroup differences between immune response variables between D0-D42 and D42-D112 were evaluated with a Wilcoxon matched-pairs signed rank test. Differences between the Control and Vaccine groups in healing time of the initial ulcer event, cumulative corneal ulcer surface area, and D0, D42, and D112 immune response variables were evaluated with a Mann-Whitney test. A value of $P < 0.05$ was considered significant. Statistical analyses were performed using GraphPad Prism (version 9.5.1 for Windows, GraphPad Software, San Diego, California; www.graphpad.com).

3. Results

Baseline herd information is summarized in Table 1. Eighty-nine Control group and 88 Vaccine group steers were enrolled. Angus, Hereford, Charolais, Red Angus, and crossbred beef steers were represented in both groups. Day 7 body weights were similar between groups. Three steers were excluded from the final dataset due to requirements for antibiotic treatment for bovine respiratory disease (n = 1), incomplete observation data after being lost to follow-up (n = 1), and death (n = 1; presumptive pneumonia). The final dataset reflected results from 87 steers in each study group.

Outcome variables assessing efficacy of the intranasal vaccine used in this study are presented in Table 2. The cumulative proportion of steers with ulcerated eyes at week 8 was less in the Vaccine group (25%) compared to the Control group (31%); however, this difference was not significant ($P = 0.399$). By week 16 (study day 112), IBK had developed in 34 steers from each group. The median healing time of the initial corneal ulcer event was 14 and 17.5 days for IBK-affected steers in the Vaccine and Control groups, respectively, but these differences were not significant ($P = 0.410$). The median overall cumulative corneal ulcer surface area for IBK-affected steers was 0.09 and 0.15 cm^2 in the Vaccine and Control groups, respectively; however, this difference was not significant ($P = 0.136$). The proportions of IBK-affected steers that required treatments with oxytetracycline, flunixin meglumine, or florfenicol were all lower for steers in the Vaccine group, but the differences from the Control group were not significant (see Table 2). Mean body weight changes between D7 and D112 were similar between groups. IBK-affected steers in the Vaccine group had significantly more observation weeks with a low maximal corneal ulcer score (CUS 0 or 1 versus 2 or 3) compared to IBK-affected steers in the Control group ($P = 0.048$).

For both Control and Vaccine group steers, serum cytotoxin-specific IgG and serum cytotoxin neutralizing antibody titers were significantly higher on D42 versus D0; however, no significant differences were observed between Vaccine and Control groups on D0, D42, or D112 in these same variables (see Table 3 and Figs. 1 and 2). Within group comparisons for tear cytotoxin-specific IgA in both the Control and Vaccine groups showed significantly lower median values on D42 versus D0, but significantly higher median values on D112 versus D42. Tear cytotoxin-specific IgA in the Vaccine group was significantly higher than the Control group on D112 ($P = 0.039$; see Table 3 and Fig. 3). Tear cytotoxin-specific IgG was significantly higher within Vaccine group and within Control group steers on D112 compared to D42; however, no differences between groups were found in tear cytotoxin-specific IgG on D42 or D112 (see Table 3 and Fig. 4). Tear cytotoxin neutralizing titers were significantly higher on D112 versus D42 for both Vaccine ($P = 0.008$) and Control group ($P = 0.002$) steers (See Table 3 and Fig. 5), but no significant differences were observed between groups in tear cytotoxin neutralizing titers on D0, D42, or D112.

4. Discussion

In this randomized controlled field trial, the efficacy of an experimental intranasal vaccine comprised of a recombinant *M. bovis* cytotoxin subunit adjuvanted with polyacrylic acid to prevent IBK in cattle was evaluated. In addition to our primary variable of interest, which was the proportion of Vaccine and Control group animals that developed IBK during the 16-week trial, secondary variables were also considered to determine if the experimental vaccine had any effects on disease severity. The secondary variables included healing time of initial ulcers, overall cumulative ulcer area over the duration of the study, proportions of IBK-affected animals that required an antibiotic treatment or flunixin meglumine (for pain), and number of weeks that IBK-affected animals experienced lower vs more severe maximal weekly corneal ulcer scores. We also evaluated tear and blood samples to measure quantitative and qualitative immune responses in a subset of animals from each of the study groups.

The results showed that while the experimental vaccine did not reduce the incidence of IBK, it reduced ulcer severity associated with IBK. This conclusion was supported by the significantly higher number of observation weeks with a lower corneal ulcer score (0 or 1 versus 2 or 3) in IBK-affected steers in the Vaccine group versus the Control group. Also supporting this conclusion was the finding that median initial ulcer healing time of Vaccine group steers was 3.5 days less versus the Control group, and the median cumulative ulcer area across the 16-week study period was lower in the group of steers that received the experimental vaccine. Furthermore, the proportions of IBK-affected animals that required treatment with an antibiotic or flunixin meglumine were numerically lower in Vaccine group animals. While these other differences were not statistically significant, collectively they suggest that steers in the Vaccine group steers experienced less severe IBK compared to steers in the Control group.

The rationale to evaluate a mucosal route of vaccination to boost ocular IgA against *M. bovis* antigens arose from previous research that demonstrated that calves treated with hydroxyurea to decrease neutrophil counts had more shallow corneal ulcers compared to control group calves following experimental *M. bovis* infections [40]. Investigators of that study postulated that neutrophils attracted to the infected eye might augment corneal stromal liquefaction following infiltration of *M. bovis* into the cornea of the infected eye. Accordingly, mucosally administered vaccines designed to elicit an IgA response in the eye could offer advantages over parenterally-administered IBK vaccines which boost plasma and tear IgG levels, and potentially might lead to unwanted neutrophil activation in the corneal stroma. In cattle, tear IgG is derived from plasma [41], and in a previous study in which the same *M. bovis* cytotoxin subunit was administered subcutaneously in cattle, increases in cytotoxin-specific tear IgG were highly correlated with increases in cytotoxin-specific serum IgG [30]. Evaluated together, these findings suggest that mucosal vaccination to stimulate a tear IgA response against *M. bovis* antigens could offer advantages over parenteral vaccination in terms of reduced ulcer severity given that IgA does not fix complement and should, in theory, result in less attraction of neutrophils into eyes due to C3a and C5a release following binding of IgG to *M. bovis* antigens in an eye affected with IBK.

In the subset of animals from which tears and serum were collected for assessment of humoral and mucosal immune responses to the *M. bovis* cytotoxin, there were no significant differences between study groups in serum cytotoxin-specific IgG or serum cytotoxin neutralizing

Table 1
Baseline herd information for a randomized controlled field study in which beef steers were vaccinated intranasally with either adjuvant alone (Control) or a recombinant *M. bovis* cytotoxin subunit (Vaccine) on D0 and D21.

Variable	Control	Vaccine	*P*
Total number enrolled	89	88	
Total number in final dataset	87[a]	87[a]	
Breed distribution in final dataset (no. of animals)			
Angus	37	38	
Hereford	3	7	
Charolais	11	2	
Red Angus	5	7	
Crossbred	31	33	
Day 7 wt (kg) (mean (standard error)) [b]	290.2 (2.5) n = 86	288.1 (2.9) n = 87	0.582

[a] Data for three steers was excluded from the final dataset for the following reasons: one control group steer required antibiotics to treat severe footrot; one control group steer had incomplete data as it was lost to follow-up for 2 consecutive weekly herd visits; and one vaccine group steer died between the 2nd and 3rd weeks of the trial (presumptive pneumonia).
[b] Day 7 wt was not recorded for one control group steer.

titers. However, we observed significantly higher serum cytotoxin-specific IgG and serum cytotoxin neutralizing titers within Vaccine group and within Control group animals between day 0 and day 42. It is possible that these increases reflect development of a systemic immune response to *M. bovis* cytotoxin following natural exposure to *M. bovis* cytotoxin. In this study, IBK had developed in both groups by study day 42, suggesting that exposure to *M. bovis* had occurred between day 0 and 42. Indeed, there were other risk factors that could have promoted spread of *M. bovis* during this timeframe, notably flies and plant awns. Fold changes in systemic antibody titers to *M. bovis* hemolysin (cytotoxin) were previously reported in cattle even in the absence of clinical IBK, suggesting that animals might naturally develop immune responses to *M. bovis* antigens such as hemolysin (cytotoxin) following natural exposure [38]. Documenting such exposure would have required regular sampling and culturing of ocular secretions of animals from which blood samples were collected, but such testing was not done as part of our study because IBK is generally considered to be a clinical diagnosis and one that veterinarians and cattle producers will often implement treatment for in the absence of culture data. Nevertheless, such culture information may have provided additional information that could have helped in interpreting immune response variables and overall study outcomes.

We expected to observe increased tear IgA in response to the vaccine antigen in vaccinated animals. Because IgA is an important constituent of tear fluid, we also expected to observe increases in tear cytotoxin neutralizing titers following vaccination. While we observed a numerical increase in median tear cytotoxin neutralizing titer in Vaccine group animals between day 0 and 42, we observed a reduction in cytotoxin-specific tear IgA between day 0 and 42 in both Vaccine and Control group animals. The exact cause of this reduction is unknown; however, it may be related to differences in tear protein concentrations on day 0 compared to days 42 and 112 in both study groups. In particular, day 0 tear protein concentrations were found to be significantly higher on day 0 versus days 42 and 112 in both study groups (data not shown). It is possible that other constituents of tear fluid could have affected the ELISA assay and that these constituents were not adequately accounted for by total protein alone which is what we used to standardize tear volume used in the ELISA assays. In contrast to day 0 tear protein concentrations, the day 42 and 112 tear protein concentrations were not significantly different between groups, and so the day 42 vs 112 comparison of cytotoxin-specific tear IgA may be a more valid comparison, assuming that other factor(s) in tears affected ELISA results. While median cytotoxin-specific tear IgA concentrations were not significantly higher in Vaccine group animals on day 42, they were significantly higher than the Control group on day 112, suggesting that this experimental vaccine affected tear cytotoxin-specific IgA concentrations.

Significant differences were also observed between some of the day 0 vs day 42 and day 42 vs day 112 immune response variables in the Control group (Table 3). Because this was a field study in which animals were likely to be naturally exposed to *M. bovis*, such responses in Control group animals may have been the result of natural exposure. In our

Table 2
Summary of outcome variables assessing efficacy of an intranasal recombinant *Moraxella bovis* cytotoxin vaccine in 87 control group and 87 vaccine group steers.

Variable		Control	Vaccine	*P*
Cumulative No. (proportion) of steers that developed IBK at week 8		27 (0.31)	22 (0.25)	0.399
Cumulative No. (proportion) of steers that developed IBK at week 16		34 (0.39)	34 (0.39)	>0.999
Healing time (days) for initial ulceration event (median (95% CI))		17.5 (14.0, 28.0) n = 34	14.0 (14.0, 21.0) n = 33[a]	0.410
Sum of individual animal ulcer SAMs (cm^2; left and right eyes combined) through D112 (median (95% CI))		0.15 (0.09, 0.45) n = 34	0.09 (0.07, 0.27) n = 34	0.136
No. (proportion) of IBK-affected steers that were treated with oxytetracycline		14 (0.41) n = 34	10 (0.29) n = 34	0.310
No. (proportion) of IBK-affected steers that were treated with flunixin meglumine		13 (0.38) n = 34	8 (0.24) n = 34	0.189
No. (proportion) of IBK-affected steers that were treated with florfenicol		4 (0.12) n = 34	1 (0.03) n = 34	0.163
Body weight change from D7 to D112 (kg) (mean (SE))		75.7 (1.7) n = 86[b]	75.5 (1.5) n = 87	0.946
No. of animal observation weeks amongst IBK affected steers with a maximum weekly CUS:	0 or 1	515	528	0.048
	2 or 3	29	16	

[a] *Note*: excludes data from one steer with an initial ulcer event on the final observation day.
[b] Day 7 wt was not recorded for one control group steer.

Table 3
Immune response variables for Control and Vaccine group steers on day 0, 42, and 112.

Immune response variable	Control[a]					Vaccine[a]					Control vs Vaccine		
	D0	D42	D112	D0-42 (*P*)[b]	D42-112 (*P*)[b]	D0	D42	D112	D0-42 (*P*)[b]	D42-112 (*P*)[b]	D0 (*P*)[c]	D42 (*P*)[c]	D112 (*P*)[c]
Serum cytotoxin-specific IgG (ng/ml)	16,006 (10,984, 24,101)	29,196 (8,517, 46,408)	33,150 (23,286, 42,597)	<0.001	0.877	17,293 (11,962, 24,363)	40,868 (23,252, 60,706)	38,015 (25,895, 58,761)	<0.001	0.784	0.707	0.185	0.128
Serum cytotoxin neutralizing titer	8.0 (4.0, 10.1)	8.0 (8.0, 16.0)	16.0 (8.0, 32.0)	0.012	0.061	8.0 (4.0, 16.0)	16.0 (16.0, 16.0)	16.0 (16.0, 32.0)	<0.001	0.221	0.711	0.133	0.823
Cytotoxin-specific tear IgA (ng/ml)	232 (61, 504)	168 (49, 234)	461 (146, 1,172)	0.048	<0.001	290 (55, 764)	164 (71, 264)	763 (485, 1,349)	0.005	<0.001	0.801	0.579	0.039
Cytotoxin-specific tear IgG (ng/ml)	55 (35, 85)	42 (20, 107)	68 (43, 185)	0.127	<0.001	53 (35, 107)	46 (30, 97)	80 (59, 183)	0.497	0.048	0.724	0.390	0.579
Tear cytotoxin neutralizing titer	2.0 (1.0, 4.0)	2.0 (1.0, 8.0)	4.0 (4.0, 8.0)	0.181	0.002	2.0 (1.0, 4.0)	4.0 (2.0, 4.0)	8.0 (4.0, 8.0)	0.094	0.008	0.663	0.317	0.303

[a] Values are reported as median (95% confidence interval); serum data represent results from 24 Control and 26 Vaccine group animals; tear data represent results from 13 Control and 13 Vaccine group animals.
[b] Intragroup D0 vs D42 and D42 vs D112 comparisons using Wilcoxon matched-pairs signed rank test.
[c] Intergroup Control vs Vaccine group comparisons on D0, D42, and D112 using Mann Whitney test.

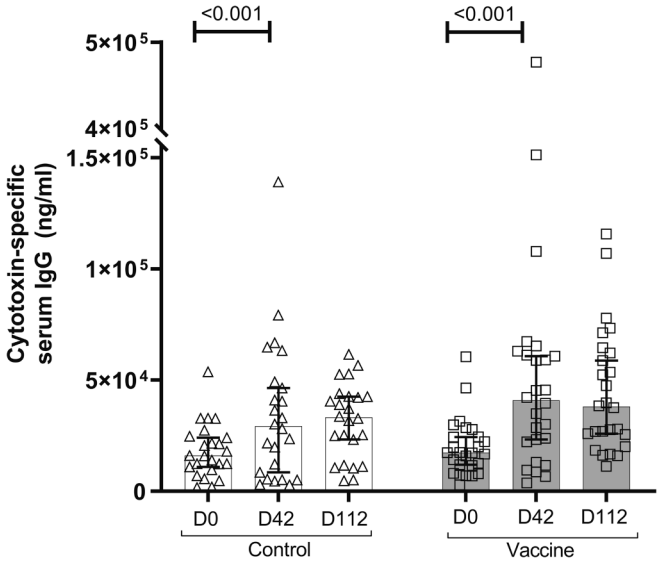

Fig. 1. Individual and group median values for serum cytotoxin-specific IgG on days 0, 42, and 112 in 24 Control group and 26 Vaccine group steers. Vaccines were administered on day 0 and 21. Error bars represent 95% confidence intervals for the median.

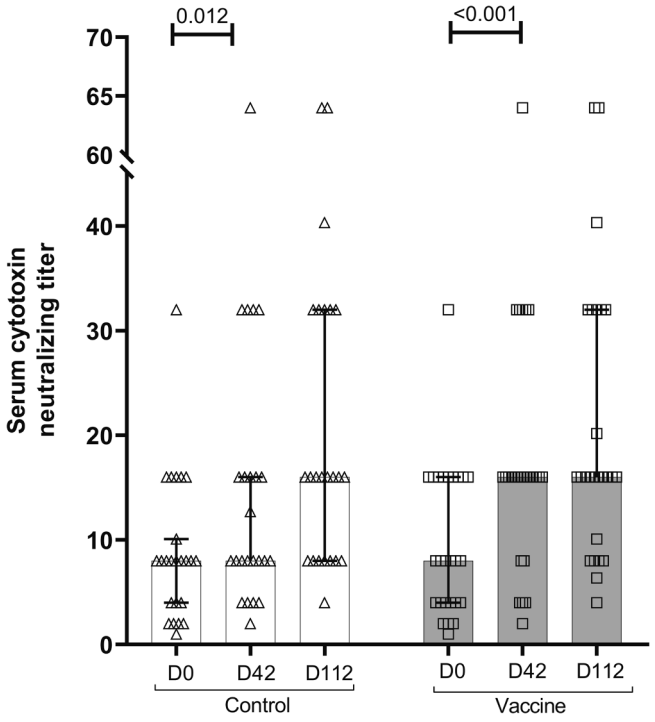

Fig. 2. Individual and group median values for serum cytotoxin neutralizing titers on days 0, 42, and 112 in 24 Control group and 26 Vaccine group steers. Vaccines were administered on day 0 and 21. Error bars represent 95% confidence intervals for the median.

statistical analyses for evaluating immune responses in the subset of animals from which blood and tears were collected, we elected to include IBK-affected as well as IBK non-affected animals. While including only non-affected animals might have helped reduce chances for an immune response following clinical IBK from skewing results, it could have also biased our results if it is assumed that an immune response to *M. bovis* cytotoxin may confer some protection against IBK. However, protective antigens of *M. bovis* could also include other components such as pilin that were not included in this experimental subunit vaccine.

Multiple published reports of randomized controlled field trials testing parenterally administered autogenous and/or commercially available vaccines designed to elicit antibody responses against *M. bovis* and/or *M. bovoculi* antigens concluded that these vaccines were not effective at preventing IBK in cattle [24–27]. One recent study reported numerically lower but not significantly lower cumulative incidence rates of IBK and IBK retreatment rates in animals vaccinated with a custom autogenous vaccine containing *M. bovis*, *M. bovoculi*, and *Mycoplasma bovoculi* antigens [42]. A previous study that evaluated the same recombinant *M. bovis* cytotoxin subunit as was used in this study, but administered parenterally with ISCOM matrices as adjuvant, found that the lowest cumulative proportion of ulcerated calves occurred in the

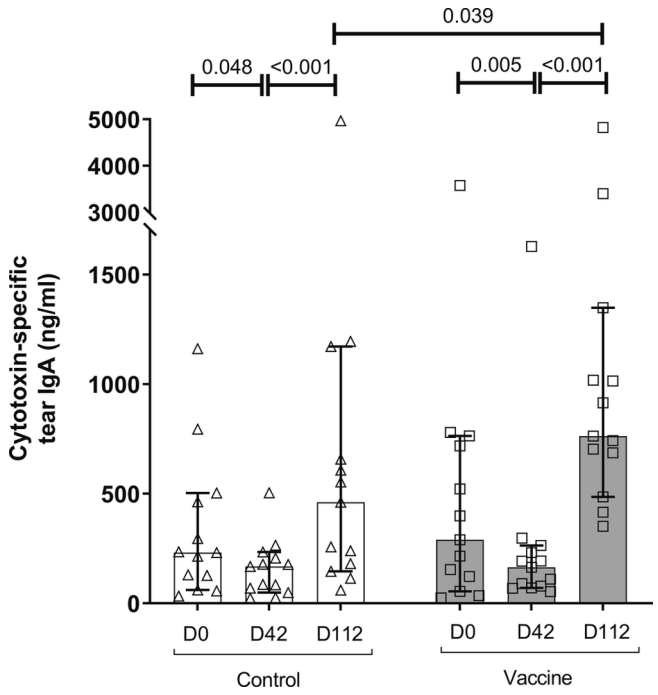

Fig. 3. Individual and group median values for tear cytotoxin-specific IgA on days 0, 42, and 112 in 13 Control group and 13 Vaccine group steers. Vaccines were administered on day 0 and 21. Error bars represent 95% confidence intervals for the median.

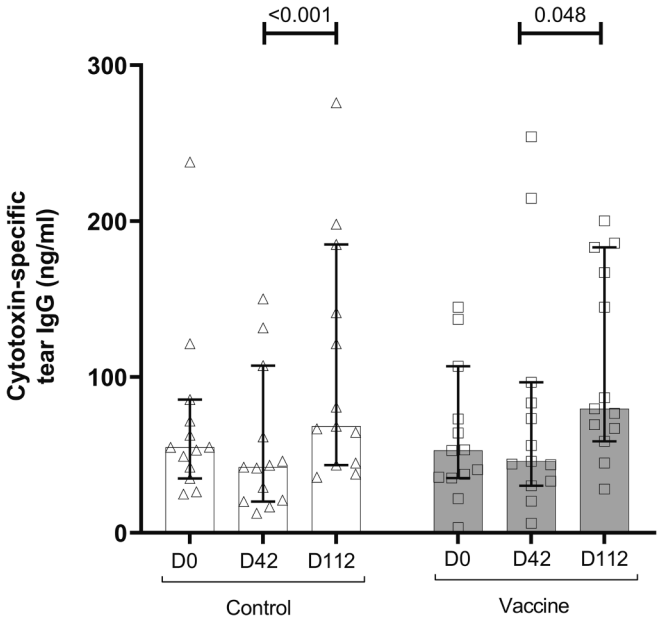

Fig. 4. Individual and group median values for tear cytotoxin-specific IgG on days 0, 42, and 112 in 13 Control group and 13 Vaccine group steers. Vaccines were administered on day 0 and 21. Error bars represent 95% confidence intervals for the median.

group that received the recombinant cytotoxin vaccine; these differences were significant at week 12, but not at week 16, and animals in this group had numerically (but not statistically significantly lower) corneal ulcer healing times [30]. Future studies need to be conducted to compare parenterally administered vaccines designed to prevent IBK with intranasally administered vaccines to determine whether intranasal administration of vaccines to prevent IBK offer advantages over

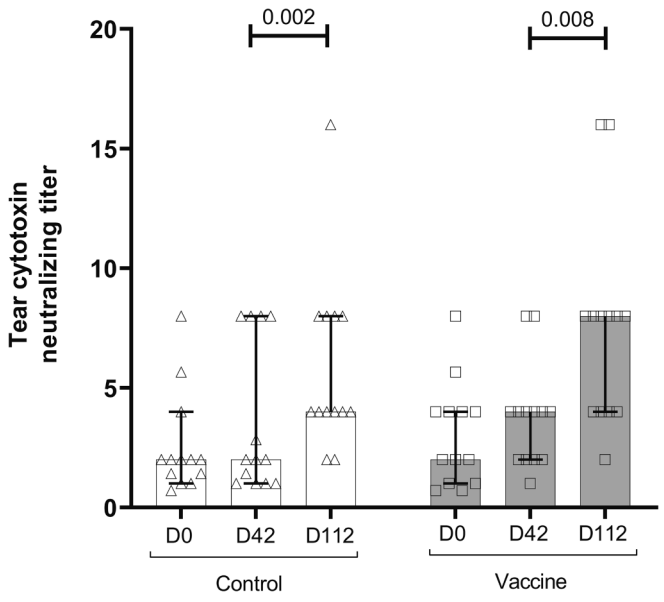

Fig. 5. Individual and group median values for tear cytotoxin neutralizing titers on days 0, 42, and 112 in 13 Control group and 13 Vaccine group steers. Vaccines were administered on day 0 and 21. Error bars represent 95% confidence intervals for the median.

parenteral delivery.

A recent study reported the existence of two different genotypes of *M. bovis* isolated from cattle, and the presence of 3 different variants of cytotoxin/hemolysin amongst these 2 genotypes [43]. Comparison of the cytotoxin subunit (amino acids 590–927) that was used as the vaccine antigen in the present study to the corresponding region of these 3 cytotoxin/hemolysin variants showed that the cytotoxin subunit antigen used in this study was identical to variants 2 and 3, and was 97.6% identical in amino acid sequence to variant 1 hemolysin (data not shown). Whether cattle with IBK associated with the variant 1 hemolysin mount an immune response that can neutralize native variant 2 or 3 hemolysins is currently unknown; however, given the high degree of identity in amino acid sequence between the 3 variants and the carboxy terminus cytotoxin subunit used in this study, we anticipate that cross reactivity is likely and that the vaccine antigen used in the present study would be effective against *M. bovis* expressing other cytotoxin/hemolysin variants.

A limitation of this study that makes it difficult to assess relative performance of this vaccine compared to previously evaluated commercial or autogenous vaccine products is that the 4-point ulcer scoring systems used in this study (score 0, 1, 2, and 3) differed from the 5-point scale used by other investigators. Our scoring system was based on maximal corneal ulcer diameter and presence of corneal perforation; other studies have utilized a 5-point scale based on overall area of affected cornea [24,27]. The scoring system used in this study was chosen in order to be consistent with the scoring system used in our previous vaccine efficacy studies. Use of a uniform scoring system by researchers investigating interventions for IBK has been suggested as a way of maximizing information obtained across multiple studies [44].

A small number of published studies have reported on the use of mucosally administered *M. bovis* antigens to prevent IBK. One study reported that an *M. bovis* vaccine enriched in fimbrial antigens administered by the aerosol route or by a combination of aerosol route followed by parenteral injection was effective against IBK [31]. A related study by the same author reported that aerosol followed by parenteral injection of *M. bovis* antigens in pregnant cows and subsequent aerosol vaccination of calves from these cows appeared to reduce the prevalence and severity of IBK compared to animals that received a control vaccine [32]. An anti-*M. bovis* pilus antigen administered intranasally in calves

was effective at increasing anti-pili IgA responses, but this response did not appear to correlate with protection against IBK [33]. Ocular administration (via spray application) of an *M. bovis* bacterin plus IL-2 and IFN-α appeared to decrease rates of IBK and disease severity in experimentally infected calves [34]. Additional research is required to determine if the vaccine used in the present study offers advantages over the same antigen administered parenterally and/or if it offers advantages over existing commercially available or autogenous parenterally administered IBK vaccines.

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Declaration of authorship

All authors attest they meet the ICMJE criteria for authorship.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: John Angelos reports financial support was provided by Russell L. Rustici Rangeland and Cattle Research Endowment.

Data availability

Data will be made available on request.

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References

[1] Henson JB, Grumbles LC. Infectious bovine keratoconjunctivitis. I. Etiology. Am J Vet Res 1960;21:761–6.
[2] Angelos JA, Spinks PQ, Ball LM, George LW. *Moraxella bovoculi* sp. nov., isolated from calves with infectious bovine keratoconjunctivitis. Int J Syst Evol Microbiol 2007;57:789–95.
[3] Gould S, Dewell R, Tofflemire K, Whitley RD, Millman ST, Opriessnig T, et al. Randomized blinded challenge study to assess association between *Moraxella bovoculi* and Infectious Bovine Keratoconjunctivitis in dairy calves. Vet Microbiol 2013;164:108–15.
[4] Loy JD, Brodersen BW. *Moraxella* spp. isolated from field outbreaks of infectious bovine keratoconjunctivitis: a retrospective study of case submissions from to 2013. J Vet Diagn Invest. 2010;2014(26):761–8.
[5] Schnee C, Heller M, Schubert E, Sachse K. Point prevalence of infection with *Mycoplasma bovoculi* and *Moraxella* spp. in cattle at different stages of infectious bovine keratoconjunctivitis. Vet J 2015;203:92–6.
[6] Levisohn S, Garazi S, Gerchman I, Brenner J. Diagnosis of a mixed mycoplasma infection associated with a severe outbreak of bovine pinkeye in young calves. J Vet Diagn Invest 2004;16:579–81.
[7] George LW, Ardans A, Mihalyi J, Guerra MR. Enhancement of infectious bovine keratoconjunctivitis by modified-live infectious bovine rhinotracheitis virus vaccine. Am J Vet Res 1988;49:1800–6.
[8] Arends JJ, Wright RE, Barto PB, Lusby KS. Transmission of *Moraxella bovis* from blood agar cultures to Hereford cattle by face flies (Diptera: Muscidae). J Econ Entomol 1984;77:394–8.
[9] Glass Jr HW, Gerhardt RR. Recovery of *Moraxella bovis* (Hauduroy) from the crops of face flies (Diptera: Muscidae) fed on the eyes of cattle with infectious bovine keratoconjunctivitis. J Econ Entomol 1983;76:532–4.
[10] Glass Jr HW, Gerhardt RR. Transmission of *Moraxella bovis* by regurgitation from the crop of the face fly (Diptera: Muscidae). J Econ Entomol 1984;77:399–401.
[11] Kopecky KE, Pugh Jr GW, Hughes DE. Wavelength of ultraviolet radiation that enhances onset of clinical infectious bovine keratoconjunctivitis. Am J Vet Res 1980;41:1412–5.
[12] Moore LJ, Rutter JM. Attachment of *Moraxella bovis* to calf corneal cells and inhibition by antiserum. Aust Vet J 1989;66:39–42.
[13] Ruehl WW, Marrs C, Beard MK, Shokooki V, Hinojoza JR, Banks S, et al. Q pili enhance the attachment of *Moraxella bovis* to bovine corneas in vitro. Mol Microbiol 1993;7:285–8.
[14] Annuar BO, Wilcox GE. Adherence of *Moraxella bovis* to cell cultures of bovine origin. Res Vet Sci 1985;39:241–6.
[15] Moore LJ, Lepper AW. A unified serotyping scheme for *Moraxella bovis*. Vet Microbiol 1991;29:75–83.
[16] Marrs CF, Ruehl WW, Schoolnik GK, Falkow S. Pilin-gene phase variation of *Moraxella bovis* is caused by an inversion of the pilin genes. J Bacteriol 1988;170:3032–9.
[17] Lepper AW, Atwell JL, Lehrbach PR, Schwartzkoff CL, Egerton JR, Tennent JM. The protective efficacy of cloned *Moraxella bovis* pili in monovalent and multivalent vaccine formulations against experimentally induced infectious bovine keratoconjunctivitis (IBK). Vet Microbiol 1995;45:129–38.
[18] Angelos JA, Hess JF, George LW. Cloning and characterization of a *Moraxella bovis* cytotoxin gene. Am J Vet Res 2001;62:1222–8.
[19] Beard MK, Moore LJ. Reproduction of bovine keratoconjunctivitis with a purified haemolytic and cytotoxic fraction of *Moraxella bovis*. Vet Microbiol 1994;42:15–33.
[20] Gray JT, Fedorka-Cray PJ, Rogers DG. Partial characterization of a *Moraxella bovis* cytolysin. Vet Microbiol 1995;43:183–96.
[21] Kagonyera GM, George LW, Munn R. Cytopathic effects of *Moraxella bovis* on cultured bovine neutrophils and corneal epithelial cells. Am J Vet Res 1989;50:10–7.
[22] Angelos JA, Ball LM. Relatedness of cytotoxins from geographically diverse isolates of *Moraxella bovis*. Vet Microbiol 2007;124:382–6.
[23] Ostle AG, Rosenbusch RF. Immunogenicity of *Moraxella bovis* hemolysin. Am J Vet Res 1985;46:1011–4.
[24] Funk L, O'Connor AM, Maroney M, Engelken T, Cooper VL, Kinyon J, et al. A randomized and blinded field trial to assess the efficacy of an autogenous vaccine to prevent naturally occurring infectious bovine keratoconjunctivis (IBK) in beef calves. Vaccine 2009;27:4585–90.
[25] Kneipp M, Green AC, Govendir M, Laurence M, Dhand NK. A randomised control trial to evaluate the effectiveness of a commercial vaccine for pinkeye in Australian beef cattle. Prev Vet Med 2023;210:105815.
[26] O'Connor A, Cooper V, Censi L, Meyer E, Kneipp M, Dewell G. A 2-year randomized blinded controlled trial of a conditionally licensed *Moraxella bovoculi* vaccine to aid in prevention of infectious bovine keratoconjunctivitis in Angus beef calves. J Vet Int Med / Am Coll Vet Int Med 2019;33:2786–93.
[27] O'Connor AM, Brace S, Gould S, Dewell R, Engelken T. A randomized clinical trial evaluating a farm-of-origin autogenous *Moraxella bovis* vaccine to control infectious bovine keratoconjunctivis (pinkeye) in beef cattle. J Vet Int Med / Am Coll Vet Int Med 2011;25:1447–53.
[28] George LW, Borrowman AJ, Angelos JA. Effectiveness of a cytolysin-enriched vaccine for protection of cattle against infectious bovine keratoconjunctivitis. Am J Vet Res 2005;66:136–42.
[29] Angelos JA, Bonifacio RG, Ball LM, Hess JF. Prevention of naturally occurring infectious bovine keratoconjunctivitis with a recombinant *Moraxella bovis* pilin-*Moraxella bovis* cytotoxin-ISCOM matrix adjuvanted vaccine. Vet Microbiol 2007;125:274–83.
[30] Angelos JA, Hess JF, George LW. Prevention of naturally occurring infectious bovine keratoconjunctivitis with a recombinant *Moraxella bovis* cytotoxin-ISCOM matrix adjuvanted vaccine. Vaccine 2004;23:537–45.
[31] Misiura M. Estimation of fimbrial vaccine effectiveness in protection against keratoconjunctivitis infectiosa in calves considering different routes of introducing vaccine antigene. Arch Vet Pol 1994;34:177–86.
[32] Misiura M. Keratoconjunctivitis infectiosa in calves–attempt at elimination by active immunization. Arch Vet Pol 1994;34:187–94.
[33] Zbrun MV, Zielinski GC, Piscitelli HC, Descarga C, Urbani LA, Defain Tesoriero MV, et al. Evaluation of anti-*Moraxella bovis* pili immunoglobulin-A in tears following intranasal vaccination of cattle. Res Vet Sci 2012;93:183–9.
[34] di Girolamo FA, Sabatini DJ, Fasan RA, Echegoyen M, Vela M, Pereira CA, et al. Evaluation of cytokines as adjuvants of infectious bovine keratoconjunctivitis vaccines. Vet Immunol Immunopathol 2012;145:563–6.
[35] Angelos JA, Edman JM, Chigerwe M. Ocular immune responses in steers following intranasal vaccination with recombinant *Moraxella bovis* cytotoxin adjuvanted with polyacrylic acid. Clin Vacc Immunol: CVI 2014;21:181–7.
[36] Angelos JA, Chigerwe M, Edman JM, Hess JF. Systemic and ocular immune responses in cattle following intranasal vaccination with precipitated or partially solubilized recombinant *Moraxella bovis* cytotoxin adjuvanted with polyacrylic acid. Am J Vet Res 2016;77:1411–8.
[37] Dohoo IR, Martin W, Stryhn HE. Veterinary Epidemiologic Research. Charlottetown, Prince Edward Island, Canada: AVC Inc.; 2003.
[38] Angelos JA, Gohary KG, Ball LM, Hess JF. Randomized controlled field trial to assess efficacy of a *Moraxella bovis* pilin-cytotoxin-*Moraxella bovoculi* cytotoxin subunit vaccine to prevent naturally occurring infectious bovine keratoconjunctivitis. Am J Vet Res 2012;73:1670–5.
[39] Corbeil LB, Anderson ML, Corbeil RR, Eddow JM, BonDurant RH. Female reproductive tract immunity in bovine trichomoniasis. Am J Reprod Immunol 1998;39:189–98.

[40] Kagonyera GM, George LW, Munn R. Light and electron microscopic changes in corneas of healthy and immunomodulated calves infected with *Moraxella bovis*. Am J Vet Res 1988;49:386–95.
[41] Pedersen KB. The origin of immunoglobulin-G in bovine tears. Acta Pathol Microbiol Scand [B]. Microbiol Immunol 1973;81:245–52.
[42] Hille MM, Spangler ML, Clawson ML, Heath KD, Vu HLX, Rogers RES, et al. A five year randomized controlled trial to assess the efficacy and antibody responses to a commercial and autogenous vaccine for the prevention of infectious bovine keratoconjunctivitis. Vaccines (Basel) 2022;10.
[43] Wynn EL, Hille MM, Loy JD, Schuller G, Kuhn KL, Dickey AM, et al. Whole genome sequencing of *Moraxella bovis* strains from North America reveals two genotypes with different genetic determinants. BMC Microbiol 2022;22:258.
[44] O'Connor AM, Angelos JA, Dennis EJ, Elizalde P, Kneipp M, Loy JD, et al. Future directions for research in infectious bovine keratoconjunctivitis. Vet Clin North Am Food Anim Pract 2021;37:371–9.