

Recombinant *Moraxella bovoculi* cytotoxin-ISCOM matrix adjuvanted vaccine to prevent naturally occurring infectious bovine keratoconjunctivitis

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Abstract A randomized, blinded, controlled field trial was conducted during summer 2006 in a northern California, USA, herd of beef cattle to evaluate the efficacy of a recombinant *Moraxella bovoculi* cytotoxin subunit vaccine to prevent naturally occurring infectious bovine keratoconjunctivitis (IBK; pinkeye). A convenience sample comprised of 127 steers were administered a subcutaneous dose of either adjuvant alone (ISCOM matrices; control group) or recombinant *M. bovoculi* cytotoxin carboxy terminus adjuvanted with ISCOM matrices (MbvA group) and were boosted 21 days later. The steers were examined once weekly for 15 weeks for evidence of IBK. No significant difference in the cumulative proportion of corneal ulcerations was detected between groups. Compared to the control calves, the MbvA vaccinates had significantly higher increases in serum neutralizing titers to *M. bovoculi* hemolysin between week 0 and week 6. The prevalence of *M. bovis* isolations was higher from ulcerated eyes of calves vaccinated with MbvA as compared to control calves. Vaccination of calves against the carboxy terminus of *M. bovoculi* RTX toxin resulted in significant increases in serum hemolysin neutralizing titers and may modulate organism type cultured from ulcerated eyes of calves in herds where both *M. bovis* and *M. bovoculi* exist. Use of *M. bovoculi* antigens alone in vaccines to prevent IBK may not be beneficial in herds where IBK is associated with both *M. bovoculi* and *M. bovis*.

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Introduction

Infectious bovine keratoconjunctivitis (IBK; pinkeye) is the most common eye disease of cattle and is seen worldwide affecting both dairy and beef breeds of cattle. The clinical signs of IBK include corneal ulceration, corneal edema, ocular pain, photophobia, and lacrimation. In the worst cases the cornea can rupture leading to permanent blindness. In 1960 the Gram negative rod *Moraxella bovis* (*M. bovis*) was demonstrated to be an etiologic agent of IBK (Henson and Grumbles 1960). In addition to *M. bovis*, however, Gram negative cocci have been isolated with or without *M. bovis* from cattle with IBK (Elad et al. 1988; Fairlie 1966; Pugh and Hughes 1971; Spradbrow 1967; Wilcox 1970). Prior to 2007, Gram negative cocci isolated from eyes of cattle with IBK were likely to be identified as *Moraxella ovis*, *Moraxella ovis*-like, *Branhamella ovis*, or *Branhamella ovis*-like. The isolation and characterization of a novel species of *Moraxella* designated *Moraxella bovoculi* (*M. bovoculi*) from ulcerated eyes of beef and dairy calves with IBK has raised the question whether *M. bovis* is the only organism that causes IBK (Angelos et al. 2007c).

To date, only isolated studies have reported on the prevalence of *M. bovoculi* as compared to *M. bovis* in IBK-associated corneal ulcers. In one study, bacteria that were cultured from IBK-associated corneal ulcers were identified as Gram negative cocci in ~46% of ulcers, while only ~21% of ulcers were *M. bovis* positive (Dueger et al. 2004). Gram negative cocci from that study were subsequently identified as *M. bovoculi* (Angelos et al. 2007c). In another study *M. bovoculi* without *M. bovis* was isolated from ~51% of initial IBK-associated corneal ulcers; *M. bovis* without *M. bovoculi* was isolated from 20% of initial ulcers in that trial (Angelos et al. 2007b). *Moraxella bovoculi* has also been isolated from reindeer with infectious keratoconjunctivitis (Tryland et al. 2009). Despite the presence of *M. bovoculi* in IBK-associated ulcers, however, Koch's postulates have not been established for *M. bovoculi* and IBK. In a recently published randomized and blinded field trial, an *M. bovoculi* autogenous bacterin was not effective against IBK (Funk et al. 2009). In that study the commercially prepared *M. bovoculi* bacterin was composed of 2 isolates made during the preceding year from the same herd. There are, however, anecdotal reports of reduced IBK in herds previously vaccinated with *M. bovis* bacterins when a switch to an *M. bovoculi* bacterin is made or when an *M. bovoculi* bacterin is used in conjunction with an *M. bovis* bacterin.

Essential proteins of *M. bovis* that are necessary for pathogenicity include pilin and cytotoxin (hemolysin, cytolysin). Pili enable *M. bovis* to adhere to the corneal surface (Annar and Wilcox 1985; Moore and Rutter 1989; Ruehl et al. 1993a). The *M. bovis* cytotoxin lyses bovine neutrophils, erythrocytes, lymphocytes, and corneal epithelial cells leading to corneal ulceration (Beard and Moore 1994; Gray et al. 1995; Hoiem-Dalen et al. 1990; Kagonyera et al. 1989a; Kagonyera et al. 1989b). The *M. bovis* cytotoxin (MbxA) is an RTX (repeats in the structural toxin) toxin (Angelos et al. 2001) that resides within an RTX operon on a pathogenicity island in *M. bovis* (Angelos et al. 2003; Hess and Angelos 2006). The *M. bovis* cytotoxin is conserved between geographically diverse strains (Angelos and Ball 2007). *Moraxella bovoculi* possesses a complete RTX operon that encodes an RTX protein designated MbvA, which has 83% deduced amino acid sequence identity with MbxA (Angelos et al. 2007a).

Previous studies have examined the efficacy of partially purified native *M. bovis* cytotoxin or recombinant *M. bovis* cytotoxin to prevent IBK. A partially purified *M. bovis* cytolysin

(cytotoxin) adjuvanted with Quil A was effective at reducing IBK in vaccinated calves (George et al. 2005). Limitations to large scale production of this partially purified *M. bovis* cytolysin vaccine prompted further investigations into recombinant *M. bovis* cytolysin vaccines. In one trial the carboxy terminus of MbxA served as the vaccine antigen (Angelos et al. 2004); in a subsequent trial, the same carboxy terminus of MbxA was expressed as a fusion protein with the amino terminus of *M. bovis* pilin (Angelos et al. 2007b). In these trials, reduced occurrences of IBK amongst vaccinates as compared to control calves were reported, however, the differences between vaccinates and controls were not consistently significant. The presence of *M. bovoculi* in cultures of eyes from IBK affected calves was documented in the 2007 trial and may have negatively affected vaccine efficacy, given that both *M. bovis* and *M. bovoculi* were present in the test herd. The present study was designed to evaluate the efficacy of recombinant *M. bovoculi* MbvA to prevent naturally occurring IBK.

Materials and methods

Study population

The study population was a convenience sample of 127 Angus (n=68), Hereford (n=2), Angus-Hereford cross (n=42), and Hereford cross (n=15) calves aged 4.9–7.1 months old. Weaning weights of the calves ranged from 159 to 294 kg. The calves were maintained on mixed grass pastures and were managed as a single cohort at the University of California Sierra Foothills Field Station, Brown's Valley, CA, USA. The terrain consisted of Sierra foothill range pasture that varied from 200–400 meters above sea level. The herd historically had a high prevalence of IBK of approximately 50% of calves. The trial started on May 2, 2006 and ended on August 15, 2006. Neither the steers nor dams of these steers had been vaccinated against IBK. The experimental procedure was approved by the University of California Institutional Animal Care and Use Committee (# 06-12522).

Pre-enrollment examination and vaccination

Calves were restrained in a hydraulic squeeze chute and each eye was examined grossly for evidence of preexisting corneal disease. Calves with two normally appearing corneas were randomly assigned to either a control or vaccine group by use of a blocked randomization scheme. On the day of enrollment (day 0) and on day 21 when booster vaccinations were administered, calves received 2 ml of either immune stimulating complex (ISCOM) matrix adjuvant (control group) or recombinant *M. bovoculi* cytolysin carboxy terminus adjuvanted with ISCOM matrix (MbvA; vaccine group). The study personnel were blinded as to the contents of the vaccines that were administered. In addition, on day 0 all calves were vaccinated against respiratory viral pathogens (Bovi-Shield® Gold 5, Pfizer Animal Health, New York, NY, USA), and on day 14 all calves received a multivalent Clostridium-Mannheimia vaccine (One Shot Ultra™ 8; Pfizer Animal Health), selenium supplementation (Mu-Se®, Schering-Plough, Union, NJ, USA), and were dewormed (Dectomax® Pour-On, Pfizer Animal Health).

Post-enrollment examination

Once weekly for 15 weeks each calf was restrained in a hydraulic squeeze chute and both eyes were examined grossly for the presence of corneal opacification by one of two examiners blinded as to the vaccine type that calves had been administered. If corneal

opacification was observed, the inferior subconjunctival cul-de-sac was swabbed with a sterile Dacron swab. Such eyes were then stained with fluorescein (Sigma, St. Louis, MO, USA) and examined with a flashlight to determine if a corneal ulcer was present; if so, the swabs were further processed as described below. A corneal ulcer was considered to be associated with IBK unless it appeared to be mechanically induced (i.e., the pattern of fluorescein staining was linear or stellate), or was present in an eye from which a plant awn was removed. If a mechanically induced ulcer was still present at the next weekly observation, it was considered to be associated with IBK, and in such cases, that observation day was counted as the starting day for the ulcer. Corneal ulcer scores (CUS) were assigned according to the widest ulcer diameter as measured with a ruler held next to the affected eye: 0=no ulcer; 1=less than or equal to 5 mm; 2=greater than 5 mm; and 3=perforated corneal ulcer. Eyes with ulcers were digitally photographed (Canon EOS D30, Canon U.S.A., Inc., Lake Success, NY, USA) with a ruler held next to the eye for subsequent determination of the corneal ulcer surface area as described below. Calves with a CUS of 2 or greater were treated once with florfenicol (40 mg/kg subcutaneously; Nuflo[®], Schering-Plough Animal Health). Rubber gloves/aprons were worn by examiners and were rinsed with 1% chlorhexidine solution between calves to prevent iatrogenic ocular infections.

Bacterial culturing of ulcerated eyes

Ocular swabs collected from eyes of calves with confirmed ulcers were streaked onto 5% sheep blood agar plates that were stored at 4°C during transport back to the laboratory; these plates were subsequently streaked for isolation. Plates were incubated at 35°C for 24 hour and examined for the presence of beta hemolysis. Beta hemolytic colonies were subcultured and identified using biochemical criteria established for *M. bovis* (Weyant et al. 1996) or *M. bovoculi* (Angelos et al. 2007c).

Vaccine formulation

Growth and propagation conditions of *M. bovoculi* 237 (ATCC BAA-1259^T=CCUG 52049^T) were performed as previously described (Angelos et al. 2007a). The methods for cloning and expressing the carboxy terminus (amino acids 590 through 927) of MbvA were as described (Angelos et al. 2007a). The recombinant carboxy terminus of MbvA was purified using a protocol for purifying recombinant *M. bovis* cytotoxin (Angelos et al. 2003). The final MbvA vaccine was mixed 1:1 with ISCOM matrices that had been prepared as previously described (Angelos et al. 2004) such that the final vaccine delivered 500 µg of recombinant protein in a 2 ml dose. The ISCOM matrix solution was mixed 1:1 with phosphate buffered saline (PBS; pH 7.4) to prepare the final 2 ml control vaccine dose. All vaccinations were administered subcutaneously in the neck.

Corneal ulcer surface area measurements

Corneal ulcers in the digital images of ulcerated eyes were traced on a computer tablet (Wacom Cintiq 15X LCD tablet, Wacom Technology Corporation, Vancouver, WA, USA) using public domain image analysis software (ImageJ program; available at <http://rsbweb.nih.gov/ij>). Magnification differences between photographs were accounted for by standardizing the scale of each tracing using the ruler in each photograph. The mean of 3 tracings of each ulcer was used to calculate the corneal ulcer surface area measurement; for data analysis, the square root of the corneal ulcer surface area was used to represent the

ulcer surface area measurement (SAM). The limit of detection was 0.008 cm^2 , an area corresponding to a 1 mm diameter circle.

Hemolysis neutralization assay

Whole blood was collected by jugular venipuncture into serum separator tubes (Corvac tubes, Sherwood-Davis & Geck, St. Louis, MO, USA) on day 0, day 42, and day 105. Serum was harvested and stored at -20°C . Prior to use in hemolysis neutralization assays, serum was heat inactivated at 56°C for 1 hour. Native cytotoxin in diafiltered retentate derived from culture supernatant of *M. bovoculi* 237 was prepared using a previously described method for preparation of *M. bovis* cytotoxin (Angelos et al. 2001). The diafiltered retentate containing native *M. bovoculi* cytotoxin was stored at -80°C until use in the hemolysis neutralization assays which were performed as previously described (Angelos et al. 2007b). The final dilution of *M. bovoculi* 237 diafiltered retentate in the neutralization assays was 1:16. The dilution endpoint was defined as the last dilution at which no hemolysis was observed. The geometric mean of three sample endpoints was used as the final dilution endpoint. For statistical analyses, the day 0–42 and day 0–105 changes in serum hemolysin neutralizing titers of nonulcerated calves to native *M. bovoculi* 237 cytotoxin were evaluated.

Statistical analyses

Baseline differences between groups with respect to age at enrollment/weight at weaning/day 0 serum hemolysin neutralizing titer and breed were evaluated by the Mann-Whitney and Pearson chi square tests, respectively. The primary outcome variable that was used to evaluate vaccine efficacy was the occurrence of IBK in calves as measured by the cumulative proportion of calves ulcerated at weeks 8 and 15 (cumulative proportion ulcerated). Additional variables examined included the proportion of calves that required at least one florfenicol treatment; the median number of days until the first ulcer was observed; the median surface area measurement of the first ulcer (median SAM for first ulcer); the median peak surface area measurement of the first ulcer (median peak SAM for first ulcer); and the median number of days for the first ulcer to heal (CUS=0; median days to heal for first ulcer). Differences in the cumulative proportion of calves that developed corneal ulcers and the number of treatments for IBK were evaluated using Pearson chi-square analysis. Risk ratios with 95% confidence intervals (CI) for developing a first ulcer were calculated for the adjuvant group versus the MbvA group at weeks 8 and 15. The Mann-Whitney test was used to evaluate differences between groups with respect to median days to first ulcer, median SAM for the first ulcer, median peak SAM for the first ulcer, median days to heal for the first ulcer, and changes in day 0–42 and day 0–105 serum hemolysin neutralizing titers. To reduce possible influences of acquired immunity due to the development of IBK, changes in hemolysin neutralizing titers were evaluated in calves that remained nonulcerated through day 105. A Kaplan-Meier (KM) survival curve was constructed for the time to first ulcer occurrence; differences between KM curves were evaluated using the log-rank test. Time to first ulcer was defined as the time of enrollment (day 0) to the time of first observed IBK-associated ulcer (CUS>0). Prevalence ratios for the isolation of *M. bovis* and *M. bovoculi* were calculated for the MbvA group versus the adjuvant group. In all comparisons, a value of $P<0.05$ was considered significant; a 95% CI that did not include 1.0 was considered significant. Statistical analyses were performed with SPSS (version 16.0; SPSS Inc., Chicago, IL, USA), and StatCalc (version 3.5.1; EpiInfo; available at: <http://www.cdc.gov/epiinfo/>).

Results

Verification of randomization

No significant differences were detected between the groups with respect to age at enrollment, calf weaning weight, day 0 serum hemolysin neutralizing titer, and breed: $P=0.63$, $P=0.142$, $P=0.91$, and $P=0.64$, respectively.

Efficacy of recombinant MbvA vaccine to prevent IBK

At week 8, the cumulative proportion of ulcerated calves was 0.4 (25/62) and 0.37 (24/65) in the control and MbvA groups, respectively. At week 15, the cumulative proportion of ulcerated calves was 0.52 (32/62) and 0.45 (29/65), in the control and MbvA groups, respectively. The differences in the cumulative proportion of ulcerated calves at week 8 and week 15 were not significantly different ($P=0.69$ and 0.43 , respectively; Table 1). The risk for ulcer development in control calves by week 8 was 1.09 times more than that of the MbvA group calves; 95% CI (0.70, 1.69). The risk for ulcer development in control calves by week 15 was 1.16 times more than that of the MbvA group calves; 95% CI (0.81, 1.66). No significant differences between vaccine and control groups were observed with respect to the proportion of calves requiring at least one nuflor treatment, median SAM of the first ulcer, median peak SAM for the first ulcer, median days to heal for the first ulcer, or the median change in weight from weaning to day 105 (Table 1). Kaplan-Meier survival analysis did not show a significant difference between groups when the times to first ulcer were examined ($P=0.38$) (Fig. 1).

Systemic immune response following vaccination

The median change in the day 0–42 serum hemolysin neutralizing titers of nonulcerated calves was significantly higher in the MbvA group (4.3) versus the control group (0) ($P=0.004$) (Table 2). There was no significant difference in the median change in the day 0–105 serum hemolysin neutralizing between nonulcerated calves in the control and MbvA groups (median=0 for both groups).

Bacterial isolates from initial ulcers

From 61 initial ulcers that developed in calves of the combined vaccine groups, *M. bovis* and *M. bovoculi* were both isolated, however, the predominant organism isolated from initial ulcers was *M. bovis* (44 isolations) as compared to *M. bovoculi* (35 isolations) (Table 3). *Moraxella bovis* was 2.88 times more likely to be isolated from ulcers in calves from the MbvA group than from calves in the control group ($P=0.09$; Table 4).

Discussion

During this 15 week trial, significant differences in the cumulative proportion of calves that developed corneal ulcerations were not detected between the group that received a recombinant *M. bovoculi* cytotoxin subunit vaccine and a control group of calves that received the adjuvant (ISCOM) alone. It is possible that a recombinant *M. bovoculi* cytotoxin vaccine might be more effective in herds in which a majority of IBK cases are

Table 1 Vaccine group outcomes assessing vaccine efficacy

Outcome	Group ^a	
	Control	MbvA
cumulative proportion ulcerated wk 8	0.40	0.37
number of calves ulcerated	25	24
<i>P</i> ^b		0.69
cumulative proportion ulcerated wk 15	0.52	0.45
number of calves ulcerated	32	29
<i>P</i>		0.43
proportion requiring at least 1 florfenicol treatment ^c	0.37	0.34
total number of calves treated	23	22
<i>P</i>		0.70
median days to first ulcer (min.–max.)	42 (28–98)	42 (28–77)
N	32	29
<i>P</i>		0.70
median SAM (min.–max.) for first ulcer	0.32 (0.11–1.55)	0.34 (0.11–1.22)
N	32	29
<i>P</i>		0.97
median peak SAM (min.–max.) for first ulcer	0.19 (0.11–0.46)	0.16 (0.11–0.4)
N ^{d,e}	11	8
<i>P</i>		0.90
median days (min.–max.) to heal for first ulcer	7 (6–21)	7 (7–14)
N ^e	11	8
<i>P</i>		0.57
median weight change (min.–max.) weaning to day 105	31.4 (13.6–58.2)	29.1 (5.5–56.4)
N	62	65
<i>P</i>		0.42

^a Sample size: control, 62; MbvA, 65

^b *P* values reflect comparisons with the control group

^c expressed as total number calves receiving at least 1 florfenicol treatment/number calves in group

^d peak first ulcer surface areas could not be calculated for 1 calf due to missing photographs

^e excludes treated calves

associated with *M. bovoculi* relative to *M. bovis*. The sample size in this study was determined by the availability of steers, and it is possible that a larger sample size may have increased the chances of finding a significant difference. In order to control for environmental variables such as plant awns, face flies, and UV radiation that can predispose to IBK, the control calves and vaccinated calves were pastured as a single group. Herd level immunity amongst the MbvA vaccinated group, however, may have lowered the power of this study as the occurrence of IBK in the control calves may have been reduced.

The fundamental question of whether *M. bovoculi* is a primary pathogen in IBK has not been answered, and specific conclusions regarding the effectiveness of including recombinant *M. bovoculi* antigens in vaccines to prevent IBK remain speculative. The use of a *M. bovoculi* autogenous vaccine was recently shown to be ineffective at preventing

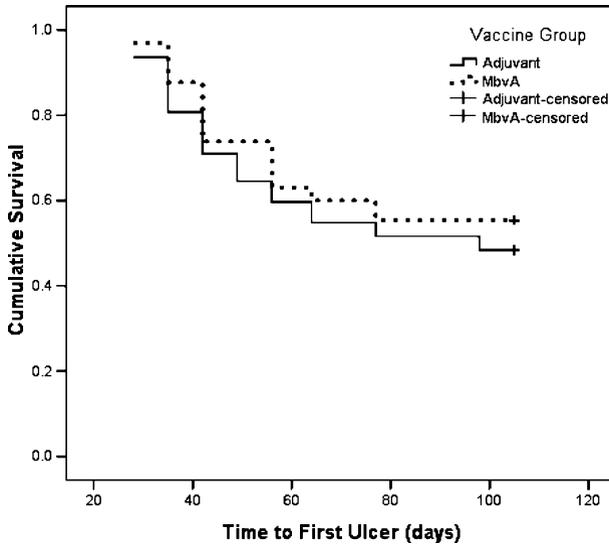


Fig. 1 Kaplan-Meier survival function curves of time to first corneal ulcer attributed to infectious bovine keratoconjunctivitis in cross-bred beef steers vaccinated with a recombinant *Moraxella bovoculi* cytotoxin vaccine (MbvA; n=65) or adjuvant alone (control; n=62)

naturally occurring IBK (Funk et al. 2009). In that study the experimental vaccine was a commercially prepared bacterin composed of two different *M. bovoculi* strains. It is possible that limited cross reactivity between the vaccine strains and *M. bovoculi* in the test herd during the trial period may have reduced the efficacy of the autogenous bacterin; cell surface antigens such as pili are considered important determinants in cross reactivity and protection against *M. bovis* (Lepper 1988). There are, however, anecdotal reports of autogenous *M. bovoculi* bacterins preventing IBK in herds when traditional *M. bovis* vaccines are not effective. These reports coupled with the occurrences of corneal ulcers from which only *M. bovoculi* can be isolated, as occurred in the present study as well as a previous study (Angelos et al. 2007b), support a hypothesis that *M. bovoculi* is important in IBK pathogenesis. If true, this hypothesis could support the rationale to include *M. bovoculi* antigens in IBK vaccines, especially in those herds where IBK has been associated with *M. bovoculi*.

Table 2 Median (min.–max.) changes from day 0–42 and 0–105 in serum hemolysin neutralizing titers of nonulcerated calves to native *M. bovoculi* cytotoxin

	Vaccine group	
	control (N=30)	MbvA (N=36)
Day 0–42	0.0 (–12.0–10.3)	4.3 (–12.0–28.0)
<i>P</i> ^a		0.004
Day 0–105	0.0 (–8.0–12.0)	0.0 (–16.0–12.0)
<i>P</i>		0.608

^a*P* value reflects the comparison with the adjuvant control group using median values (Mann-Whitney test)

Table 3 Frequency of *Moraxella* spp. isolations from 61 initial ulcers

Organism	Vaccine group		Total
	control (N=32)	MbvA (N=29)	
<i>M. bovoculi</i>	9	3	12
<i>M. bovis</i>	11	10	21
<i>M. bovoculi</i> plus <i>M. bovis</i>	9	14	23
No <i>Moraxellae</i> isolated	3	2	5

The recombinant *M. bovoculi* antigen that was used in this trial represented the carboxy terminal 338 amino acids of MbvA; in total, *mbvA* encodes a 927 amino acid protein (Angelos et al. 2007a). Previously, the analogous portion of *M. bovis* MbxA was demonstrated to elicit a hemolysis neutralizing antibody response in rabbits (Angelos et al. 2007a). Based on the results of the present study, it can be concluded that calves respond similarly against MbvA by mounting a hemolysis neutralizing antibody response. The increase in neutralizing titer between day 0–42 was significantly higher in nonulcerated vaccinated calves compared to nonulcerated control group calves. The significant increase, however, was not maintained for the duration of the trial. Previously when we measured the change in serum hemolysis neutralizing titer of non-ulcerated calves that were vaccinated with a recombinant *M. bovis* pilin-cytotoxin, we identified statistically significant increases not only in the day 0 to day 42 (3 weeks post booster vaccination) titers, but also in the day 0 to day 126 titers (Angelos et al. 2007b). It is possible that the observed difference in the duration of immune responsiveness against MbvA versus MbxA in our two studies is related to the composition of the antigen used in the neutralization assays. In a previous study that examined the stability of *M. bovis* cytotoxin (MbxA) prepared as a diafiltered retentate derived from culture supernatant, both full length MbxA as well as immunoreactive MbxA fragments were identified in the diafiltered retentate (George et al. 2004). It is possible that during the preparation of *M. bovoculi* diafiltered retentate used for the hemolysis neutralization assays, partial length fragments of MbvA were similarly copurified along with native MbvA, and that in the neutralization assays, these partial length fragments, while lacking hemolytic activity, still bound to antibodies against MbvA and rendered such antibodies unavailable to neutralize full-length native MbvA. It is also possible that other proteins copurified with MbvA in the diafiltered retentate of *M. bovoculi* culture supernatant may have served to promote hemolysis, effectively reducing the measured titers. In the hemolysis neutralizing assays that were performed in the present study, the *M. bovoculi* diafiltered retentate had less hemolytic activity relative to similarly prepared *M. bovis* diafiltered retentate that we have prepared for previous studies; as such,

Table 4 Frequency of isolation of *M. bovis* and prevalence ratio (PR) for the association of *M. bovis* with initial ulcers

<i>M. bovis</i> isolated from ulcer	Vaccine group		PR	<i>P</i>
	MbvA	control		
Yes	24	20	2.88	0.094
No	5	12		
Total	29	32		

the working dilution of the *M. bovoculi* diafiltered retentate in the neutralization assays was only 1:16. In contrast, the final working dilution of *M. bovis* diafiltered retentate in the neutralization assays in our 2007 trial was 1:128 (Angelos et al. 2007b).

It is possible that an immune response against epitopes found in the amino terminus of the *M. bovoculi* cytotoxin might improve the efficacy of a recombinant *M. bovoculi* cytotoxin vaccine. While no previous studies have examined the effectiveness of full length recombinant *M. bovis* cytotoxin to stimulate a hemolysis neutralizing immune response, one study identified the presence of hemolysis neutralizing epitopes in the amino terminus of HlyA, the RTX toxin expressed by uropathogenic *Escherichia coli* (Ji and O'Hanley 1990). As such, a full length recombinant *M. bovoculi* and/or *M. bovis* cytotoxin may improve the efficacy of recombinant *Moraxella* spp. cytotoxin based vaccine against IBK as compared to carboxy terminus cytotoxin based vaccines as reported here and in earlier studies (Angelos et al. 2007b; Angelos et al. 2004).

In this study, the prevalence of *M. bovis* from cultures of initial ulcers was nearly three times higher in the MbvA vaccinates compared to the control calves. While this higher prevalence was not statistically significant, it may have biological significance. In a previous study that evaluated a recombinant *M. bovis* pilin-cytotoxin vaccine, we found a higher prevalence of *M. bovoculi* in cultures from initial ulcers of calves vaccinated with two *M. bovis* antigens: pilin and cytotoxin (Angelos et al. 2007b). Moreover, there was a higher prevalence of *M. bovis* in ocular cultures from control calves as compared to *M. bovis* pilin-cytotoxin vaccinated calves (Angelos et al. 2007b). In the present study it is possible that the immune response against *M. bovoculi* MbvA allowed *M. bovis* to colonize the ocular surface more readily or provided some other competitive advantage relative to *M. bovoculi*. The potential for a host immune response to alter the expression of different *M. bovis* pili was previously suggested (Ruehl et al. 1993b). Additional investigations of the ocular flora of not only IBK-affected, but also normal healthy eyes of calves are necessary to help define mechanisms by which vaccination may affect the development of IBK and of the role that *M. bovoculi* plays in IBK pathogenesis.

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