

Evaluation of the ability of an alternative therapy to aid in corneal healing and reduction of pain and infection following experimentally induced Bovine Keratoconjunctivitis

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Abstract:

Thirty dairy, 8 Holstein and 20 Jersey, bull calves having determined to have normal ophthalmic examinations and culture negative for *M. bovis* were randomly assigned to 3 groups for a single eye block randomized blinded challenge study. Calves were housed in pairs according to their respective group in an approved isolation facility. Each of the calves in Groups 1 and 2 had a 0.6 mm corneal lesion made on the left central corneas utilizing n-heptanol. Immediately following lesion formation, 1.0×10^7 of *Moraxella bovis* (strain Epp63-300; origin: NADC) was administered topically to the left central corneas of Groups 1 and 2. The calves in Group 3 (Control group) received topical corneal administration of *M. bovis* to their left eyes but nothing further. In Group 1, two ml of Vetericyn Plus™ Pinkeye Spray was administered topically to each calves' cornea twice daily for 10 days. In Group 2, two ml of 0.9% saline was administered topically to each calves' cornea twice daily for 10 days. Each animal was given a pain score twice daily. All eyes were sampled for culture on day -7, 0, 1-5, and day 10. Daily fluorescein staining was performed on the eyes of all calves followed by digital photography of the lesion to assess healing of the corneas. The sizes of the lesions were assessed daily utilizing image J software. Additionally, serum and plasma samples were drawn from all calves on days 0, 1, 10, 11, and 17 and evaluated for changes in sodium and chloride levels. Urine samples, and liver, muscle, and fat biopsies were collected from all calves on days 0, 11, and 17 and evaluated for chlorine utilizing the DPD colorimetric method. Calves in group 2 only were determined to be culture positive for *M. bovis* during the study period. On average for Group 1 there was a reduction in pain score by 79.1% by day 2 and an 83.7% reduction in pain by day 10 when compared to controls. Group 2 had an average reduction in pain score of 18.3%, and 67.9% by day 2 and by day 10, respectively, when compared to controls. The average Days to cure for Group 1 was 2.2 and Group 2 was 5.5, respectively. It was found that the Days to cure was significantly different between Group 1 and Group 2 ($P = 0.0161$). The measured corneal lesion circumferences of the calves in Group 1 compared to the calves in Group 2 was significantly different ($P = 0.0375$) and the difference between days was significantly different ($P = <0.0001$). There was no appreciable difference in the amount of Na & CL in the plasma & serum samples among all three groups at any of the sampling time points ($P = <0.0001$). There was no difference between pre (Day 0) and post treatment (Day 11 and 17) in chlorine within Group 1 in the muscle, liver, fat & urine samples at any of the time points sampled in any of the calves sampled. The results of this study seem to indicate that Vetericyn Plus™ Pinkeye Spray can be utilized as an alternative therapy to significantly aid in the reduction of pain, infection and healing time of corneal lesions in calves experimentally infected with *M. bovis*.

Keywords: cattle, *Moraxella bovis*, pinkeye, residues

Introduction:

Infectious Bovine Keratoconjunctivitis (IBK), commonly called “pink eye”, is a very painful condition affecting beef and dairy cattle worldwide.¹⁶ The bacterium, *Moraxella bovis* is known to be responsible for this condition.¹⁶ It has been estimated that annual losses associated with only decreased weight gain from infected cattle exceeds 150 million dollars.^{24,31} Infectious Bovine Keratoconjunctivitis has been referred to as the most important ocular disease in cattle worldwide.^{4,24,8,34} The majority of other ocular infections seen in cattle are characterized by conjunctivitis and rarely resulting in keratitis and if present is minimal in nature. There are a number of pathogens associated with IBK in cattle, such as Bovine Herpes Virus-1 (BHV-1) which is the causative agent of Infectious Bovine Rhinotracheitis (IBR). The clinical signs of IBR are severe conjunctivitis and edema of the cornea which usually originates near the limbus and corneal ulceration is not a common finding.^{8,19} Other differentials include *Mycoplasma spp* which may also result in conjunctivitis and keratitis of cattle, either alone or in conjunction with *M. bovis*^{8,34}. The severity of *M. bovis* infection can be increased due to additional infections with other bacteria and viruses such as *Moraxella bovoculi*, *Mycoplasma spp.* and IBR.^{8,34}

M. bovis, is a gram negative rod shaped bacteria, has been considered the primary causative agent of IBK in cattle.^{5,21} In a recent study by O’Conner et al., 2012, *Moraxella bovoculi* was isolated from cattle with naturally occurring IBK.²⁷ There are reports of *M. bovoculi* in association with ocular infections in reindeer and calves.^{4,27,38} *M. bovoculi* is considered a risk factor for development of IBK.⁶ However, in a subsequent study with *M. bovoculi* and *M. bovis*, *M. bovis* only resulted in central corneal ulceration.^{5,21} Currently there are seven different serogroups of *M. bovis*.¹³ Genetic predisposition along with plant awns, face flies, ultraviolet radiation from bright sunlight, dry and dusty environmental conditions, and shipping stress are all mechanical risk factors that have been associated with development of IBK in cattle.³⁵ Also, nutritional risk such as trace mineral deficiencies such as selenium and copper deficiency can play a part in presence of disease. Appropriate management including fly control, reducing dusty feed can go a long way to reducing problems with IBK.⁸ However, in confined feeding operations and range conditions IBK is often hard to control. Vaccination has provided mixed results but can reduce clinical signs.^{7,8,13,19,25,31} Vaccination can be fairly expensive especially when the efficacy is questionable. Historically, antibiotic treatment of clinical cases has been the preferred method of therapy.⁶

However, there has been a strong push by the Centers for Disease Control (CDC), the Food and Drug Administration (FDA), the World Health Organization (WHO), and the American Veterinary Medical Association (AVMA) to develop and utilize products that do not predispose to antimicrobial resistance. Hence, the necessity for further evaluation of therapeutics such as Vetericyn™ Pink Eye Spray for use as a topical therapy for *M. bovis* ocular infection in cattle to aid in healing of corneal ulceration. Therefore, it was the focus of this paper to scientifically evaluate the therapeutic usage of Vetericyn™ Pink Eye Spray on IBK caused by *M. bovis* in cattle. It was our hypothesis that Vetericyn Plus™ Pink Eye Spray would significantly aid in inhibition and elimination of *M. bovis* from corneal lesions and in corneal healing, and reduction of pain following experimentally induced IBK. Additionally the use of Vetericyn Plus™ Pink Eye Spray would not result in any detectable tissue residues in serum, plasma, liver, fat, muscle and urine following topical ocular administration.

Materials and Methods:

Experimental Design:

Thirty dairy bull calves, eight Holstein and 22 Jersey, ranging in age from two weeks to six months of age were utilized in this Institutional Animal Care and Use Committee approved study. Two trials were performed with Trial 1 consisting of 18 total calves with 6 per group and then Trial 2 consisting of 12 total calves with 4 per group. A single eye randomized controlled challenge design was employed in this study in accordance with previous study designs.^{15,21} All animals were examined by a boarded ophthalmologist and were excluded from the study if there is any evidence of ocular abnormalities including: adnexal, conjunctival, corneal, anterior segment or posterior segment abnormalities. Thirty calves determined to have normal ophthalmic exams and culture negative for *Moraxella bovis* were utilized in the study. The calves were randomly placed into three groups, as detailed below, with 10 calves in each group. Groups 1 and 2 had corneal lesions induced in their left eyes but Group 3 functioned as the control group with no lesion formation. Topical corneal administration of *M. bovis* organisms was performed in the left eye of each calf. The left and right eyes of each calf were evaluated twice daily for evidence of pain and discharge and scored accordingly utilizing a scale of one to four. Both eyes of each calf were stained daily with fluorescein stain following pain scoring and digital images were taken on days -4, 0-10. The Digital images of the lesions were analyzed utilizing image J (National Institutes of Health, Rasband WS; <http://imagej.en.softonic.com/>). Each group functioned as a control due to the initial culture and evaluation, and again on day 0. Cultures were taken at various time points throughout the study period. A general physical exam along with evaluation of serum total protein was performed on each calf to determine if there was evidence of any underlying disease state which might lead to problems with corneal healing. Serum and plasma samples were drawn from all calves on days 0, 1, 10, 11, and 17 and evaluated for changes in sodium and chloride levels. Urine samples, and liver, muscle, and fat biopsies were collected from all calves on days 0, 11, and 17 and evaluated for chlorine utilizing the DPD colorimetric method.

Group 1 consisted of the calves in which a 0.6mm corneal lesion was induced with n-heptanol (Sigma-Aldrich, St. Louis, MO) in the left eye of each calf followed by immediate administration of *M. bovis* organisms to the left eye. The right eye served as a control. The left eye in group one was inoculated with 1×10^7 organisms (strain Epp63-300; R. Rosenbusch laboratory; origin: NADC) of *M. bovis*. Following inoculation of organisms, the left and right eyes were treated with Vetericyn Plus™ Pink Eye Spray (Innovacyn, Rialto, CA) for a total of two mL per application (3 sprays) twice daily for 10 days starting 24 hours following administration of the organisms. The right eye functioned as a control for Vetericyn Plus™ Pink Eye Spray (Innovacyn, Rialto, CA).

Group 2 consisted of the calves in which a 0.6mm corneal lesion was induced with n-heptanol (Sigma-Aldrich, St. Louis, MO) in the left eye of each calf followed by immediate administration of *M. bovis* organisms to the eye. The right eye served as a control. The left eye in group one was inoculated with 1×10^7 organisms (strain Epp63-300; R. Rosenbusch laboratory; origin: NADC) of *M. bovis*. Following inoculation of organisms, the left and right eyes were treated with 0.9% sterile saline (Baxter, Deerfield, IL) for a total of two mL per application (3 sprays) twice daily for 10 days starting 24 hours following administration of the organisms. The right eye functioned as a control for 0.9% sterile saline (Baxter, Deerfield, IL). Group 3, the control group, consisted of calves in which there was not an induced corneal lesion to either eye. However, the left eye of each calf in this group was inoculated with *M. bovis*

organisms at a concentration of approximately 1×10^7 (strain Epp63-300; R. Rosenbusch laboratory; origin: NADC) organisms of *M. bovis*. Following inoculation of organisms, the left and right eyes were not treated but functioned as controls.

Pain Scoring:

The left and right eyes of each calf were evaluated twice daily for evidence of pain and discharge and scored accordingly utilizing a scale of one to four. The eye pain/blepharospasm consisted of a scale of 1-4 with 1 = no pain (normal with no discharge and/or tearing), 2 = mild pain (intermittent partial closure of the eye lid plus or minus discharge and/or tearing), 3 = moderate pain (intermittent complete closure of the eyelid plus or minus discharge and/or tearing), 4 = severe pain (consistent complete closure of the eye lid plus or minus discharge and/or tearing). Both eyes were stained daily with fluorescein stain following pain scoring and digital images were taken on days -4, 0-10.

Housing:

All animals were housed at the AUCVM Sugg Laboratory Isolation Facility at Auburn, AL. The calves were housed in paddocks and then placed in the Sugg Laboratory Isolation facility for four days prior to the start of the study to acclimate. The calves were housed in isolation until day 11 of the study. Calves were kept in pairs according to the assigned grouping. It was imperative to keep these animals in isolation to have a controlled environment and prevent additional variables within the study. All calves were fed twice daily an 18-22% protein calf grain for the duration of the study with free choice hay and water and milk and or milk replacer as necessary if calves were not yet weaned.

Ophthalmic Examinations:

Ophthalmic examinations of all calves eyes included in the study were performed by a board certified veterinary ophthalmologist. Any calves with evidence of adnexal, conjunctival, corneal, anterior segment or posterior segment abnormalities were not included in the study. The initial ophthalmic examinations were performed on Days -7 to -4 following sedation of each calf with xylazine at a concentration of 0.05mg/kg IV (Rompun™) (Bayer, Shawnee Mission, KS). A sterile culture was taken from the cornea and conjunctiva of each eye of each calf utilizing an individual sterile culture swab [BD CultureSwab™ EZ Collection and Transport System (Franklin Lakes, New Jersey) (<http://www.bd.com/ds/productCenter/CT-CultureSwab.asp>)] for each eye. All cultures were immediately taken to the AUCVM Bacteriology Mycology Diagnostic Laboratory and analyzed for *M. bovis* and any other commensal bacteria. Only, animals negative for *M. bovis* were utilized in the study. Further ophthalmic examination of each calf included a Schirmer tear test (Merck Animal Health, Kenilworth, NJ) performed in accordance with previous descriptions.⁹ Tonometry testing was also performed on all calves using the TonoVet1 (Tiolaty, Helsinki, Finland) in accordance with previous descriptions.^{22, 28} Fluorescein staining of the cornea utilized FUL-GLO® fluorescein sodium strips USP 0.6mg (Akorn, Lakeforest, IL) in accordance with the standard procedure as detailed previously.^{23, 32, 36} Additionally, the examination included assessment of the adnexa, cornea, anterior chamber, iris, lens, and anterior vitreous through utilization of a trans illuminator and slit-lamp biomicroscope (Kowa SL-15 biomicroscope; Kowa Ophthalmic Diagnostics, Torrance, CA), and indirect ophthalmoscopy and direct ophthalmoscopy were

performed following pharmacologic pupillary dilation with Tropicamide 1% (Mydracil™) (Bausch & Lomb Incorporated, Rochester, NY) in accordance with standard procedures detailed previously.^{23, 36}

Corneal Lesions:

On day 0, the calves were anesthetized with xylazine (Rompun™) (Bayer) at a concentration of .05 mg/kg IV and 0.05mg/kg IV of butorphanol tartrate (Torbugesic®) (Zoetis, Kamamazoo, MI). The left corneas of each calf were anesthetized with topical 0.5% proparacaine hydrochloride (Bausch & Lomb Incorporated, Rochester, NY). The central corneal lesion was then created by utilizing n-heptanol (Sigma-Aldrich, St. Louis, MO).¹⁴ Corneal epithelial removal was accomplished by placing .6 cm diameter paper disc (Kimwipes®) soaked in n-heptanol (Sigma-Aldrich, St. Louis, MO) for 1 min. The paper discs were placed mid-cornea on the left cornea for a period of 2 min prior to removal of the discs. Both eyes were then fluorescein stained and photographed. The calves in Groups 1 and 2 had corneal lesions induced in their left eyes. There were no induced corneal lesions in Groups 3 but they were fluorescein stained and photographed as well. Banamine was administered as a one-time dosage at the time of corneal lesion formation in Groups 1 and 2 and prior to fluorescein staining in Group 3 at the dosage of 1.1 mg/kg IV.

***Moraxella bovis* Inoculum and Corneal Inoculation:**

Moraxella bovis strain Epp63-300; R. Rosenbusch laboratory; origin: NADC; provided by Iowa State through Dr. A. O'Conner was grown overnight on trypticase soy agar supplemented with 10% sterile bovine blood (TSAB) at 37.0° C in a 5% CO₂ atmosphere. The morning of the challenge a 0.65% saline suspension of *M. bovis* was prepared from a fresh isolate to obtain a density of 1 X10⁷ CFU/ml. To retain pathogenicity through hemolysin and pili production, the cultures were maintained with minimal *in vitro* passages.¹⁰ On Day 0, immediately following lesion formation in Groups 1 and 2 and ocular evaluation of Group 3, the left eyes of all calves were inoculated by pipetting the saline suspension containing 1 x 10⁷ organisms of *M. bovis* on to the left cornea. To confirm viability and a positive control, samples of inoculum were plated out onto TSAB prior to the challenge and then again after inoculating the calves. For the negative control, a sterile swab was streaked out on a TSAB. New gloves were used for each calf.

Cultures:

All bottles of Vetericyn Plus™ Pink Eye Spray (Innovacyn, Rialto, CO) and 0.9% sterile saline (Baxter, Deerfield, IL) were cultured prior to use to make sure that they were not contaminated. The nozzels of the sprayers were cleaned with alcohol prior to each use. A separate spray bottle was used in each isolation stall. Culture samples were collected from each calf at various time points throughout the study period on each calf on days: 0, 1-5, and 7 and 10. If the culture was determined to be negative for *M. bovis* at a sampling time point and the following culture was negative then no additional samples were collected except for day 7 and day 10. If the day 7 cultures were negative for *M. bovis* the day 10 sample was not cultured. The control animals (Group 3) and the right eyes of Groups 1 and 2 were only cultured initially days -7 to -4 and then on days 0, 1, and 7.

Image J Analysis:

Digital images were taken with a Nikon D5200 24.1 Mega Pixel Digital SLR 18-55 mm (Nikon, Melville, NY) taken from approximately 10 cm away from the cornea of both eyes on study days -4, 0-10 following fluorescein staining. Specific evaluation and measurement of corneal lesions was completed through examination of the digital picture and utilization of the Image-J software (National Institutes of Health, Rasband WS; <http://imagej.en.softonic.com/>). The image-J program had a scale tool and there was a scale in each photo taken so each image could be a scaled. This allowed for collection of accurate and comparable measurements that were able to undergo statistical comparisons. The Image-J software was used to determine the circumference, width, height, and area of all lesions on a daily basis.

Assessment of Total Protein:

On Day -7 to -4 a 10 mL blood sample was collected from all calves via jugular venipuncture into the appropriate vacutainer tubes (BD Vacutainer tubes; Becton Dickinson, Franklin Lakes, New Jersey, USA) for analysis of the calf's total protein (serum). All samples were placed in a refrigerator or cooler and transported immediately to the AUCVM Clinical Pathology Laboratory for centrifugation and assessment of total protein.

Sodium and Chloride levels:

On Days -4, 0, 1, 10, 11 and 17 all calves that were in Group 1 had serum and whole blood collected in a manner consistent with previously described. All samples were placed in a refrigerator or cooler and transported immediately to the AUCVM Clinical Pathology Laboratory for assessment of sodium and chloride levels.

Biospy Procedures:

On Days 0, 11 and 17 all calves that were in Group 1 had urine samples, two liver biopsies, two muscle biopsies, and two fat biopsies collected. The samples were collected for residue testing of Vetericyn Plus™ Pink Eye Spray. The active ingredient of Vetericyn Plus™ Pink Eye Spray is hypochlorous acid so measurement of chlorine was the best detection of a residue that might occur following its use. The samples were tested via the DPD colorimetric method for chlorine as detailed herein.

Liver Biopsy Procedure:

Calves were shaved on the right mid-thorax at the level of the 10th intercostal space and underwent aseptic preparation of the shaved area. Then, 0.5 mL of 2% lidocaine hydrochloride (IVX Animal Health, St. Joseph, MO) was administered intradermal within the sterile site and then the site was again aseptically prepared. A 15 mm Bard-Parker® scalpel blade (Aspen Surgical, Caledonia, MI), was utilized to make a stab incision through the skin in the area that was anesthetized with the lidocaine. Then utilizing a Bard-Parker® 14 gauge biopsy needle (Aspen Surgical, Caledonia, MI), two liver biopsies were collected. A total of 20-30 mg of tissue (10-12mg/biopsy) was collected in accordance with the procedure outlined by Herdt, T. 2013 (http://www.dcpah.msu.edu/sections/nutrition/WEB_CD.NUTR_REF.002.pdf). The samples were placed in labeled micro-centrifuge Eppendorf tubes (Eppendorf, Hauppauge, NY). The biopsies were then homogenized with 1 mL of sterile water following mincing tissue with a 25 gauge needle attached to a 3 mL syringe (Becton Dickinson and Co.; Franklin Lakes, NJ). They were then

vortexed with a Fisher Scientific™ Mini Vortex Mixer) (Thermo Scientific Inc, Waltham, MA) for two intervals of 60 seconds with a 5 second rest between. Each sample was then sonicated for two 60 second intervals with 5 second rest between the intervals with a Virsonic 100 Sonicator (ViTis, Gardiner, NY). The solution was then tested with the DPD colorimetric assay for chlorine.

Muscle Biopsy Procedure:

Two muscle biopsies were collected from each calf in Group 1 on Days 0, 11, and 17. The biopsies were collected from the right mid-semi-membranosus muscle following aseptic preparation. The biopsies were collected utilizing a Bard-Parker® 14 gauge biopsy needle (Aspen Surgical, Caledonia, MI). The samples were placed in labeled micro-centrifuge Eppendorf tubes (Eppendorf, Hauppauge, NY), homogenized, sonicated and assayed as in the same fashion as the liver biopsy samples as detailed above.

Fat Biopsy Procedure:

Two fat biopsies were also collected from each calf in Group 1 on Days 0, 11, and 17. The biopsies were taken at the base of the right ear in the fat pad following aseptic preparation of the area. Then, 0.5 mL of 2% lidocaine hydrochloride (IVX Animal Health, St. Joseph, MO) was administered intradermal within the sterile site and then the site was again aseptically prepared. A 15 mm Bard-Parker® scalpel blade was utilized to make a stab incision through the skin in the area that was anesthetized with the lidocaine. The fat was collected via biopsy as previously detailed and through use of a sterile hemostat which was utilized to grasp the fatty tissue. The samples were placed in labeled micro-centrifuge Eppendorf tubes (Eppendorf, Hauppauge, NY), homogenized, sonicated and assayed as in the same fashion as the liver biopsy samples as detailed above.

Urine Collection Procedure:

All calves in Group 1 had urine collected on Days 0, 11, and 17. Urine samples were collected free catch into sterile urine specimen cups (Thermo Scientific Inc, Waltham, MA) and then assayed for chlorine via the DPD colorimetric method.

DPD Colorimetric Method for Detection of Chlorine:

Urine, muscle, liver and fat samples were tested with the The N,N-diethyl-p-phenyldiamine (DPD) Colorimetric method. This method utilized a Chlorine (Free & Total) Color Disc Test Kit, Model CN-66 (Hatch®, Loveland, CO) and Chlorine Reagent Set, DPD Free and Total Chlorine (Hatch®, Loveland, CO). The samples were run in duplicate. Every sample was run with a control in accordance with the directions of the color disc test kit. The sample was placed in the sample tube provided and then q.s. to 5 mls with sterile water. The control tube was filled with 5mls of sterile water. The powdered reagent was added to the control tube and to the sample tube and swirled to mix. The vial was inserted into the meter and the intensity of the color change following addition of the reagent was read at 1 min and again at 3 min and compared to the color disc of the meter. The range of the meter was 0 – 3.4 mg/L, equivalent to 0 -3.4 ppm (parts per million). The reading was recorded for all samples. This unit was utilized to check for chlorine residues (hypochlorous acid) in plasma, serum, urine and blended liver with sterile water and blended muscle tissue with sterile water.

Statistics:

Statistical evaluation was performed utilizing SAS[®] software (SAS Institute, Cary, NC). The data were natural log transformed and a Kenwood-Roger correction was utilized. The covariance structure utilized was autoregressive and/or Toeplitz.

Results:

All calves in group 1 and 2 developed corneal lesions in the left eye as determined by fluorescein staining. All calves in group 2 developed lesions consistent with IBK in the left eyes. Calves in group 2 only were determined to be culture positive for *M. bovis* during the study period. Two calves in Group 1 and two calves in Group 2 had to be removed at the end of the study and were not included in the treatment comparisons. It was found that between Days 1 and 2, Group 1 had significantly decreased pain scores when compared to controls. Between Days 1 and 2, Group 1 had significantly, $P < 0.05$, decreased pain scores when compared to controls. On average for Group 1 there was a reduction in pain score by 79.1% by day 2 and an 83.7% reduction in pain by day 10 when compared to controls. Group 2 had an average reduction in pain score of 18.3%, and 67.9% by day 2 and by day 10, respectively, when compared to controls.

The average Days to cure for Group 1 was 2.2 and Group 2 was 5.5, respectively. It was found that the Days to cure was significantly different between Group 1 and Group 2 ($P = 0.0161$). The measured corneal lesion circumferences of the calves in Group 1 compared to the calves in Group 2 was significantly different ($P = 0.0375$) and the difference between days was significantly different ($P = <0.0001$) but interaction of treatment and day was not significantly different ($P = 0.329$). The measured corneal lesion width between the treatment groups was determined to be significantly different ($P = 0.0147$) and the difference between days was significantly different ($P = <0.0001$) but interaction of treatment and day was not significantly different ($P = 0.329$). The difference between the measured corneal lesion areas of the treatment groups trended towards significance ($P = 0.0829$) and the difference of the lesion area between days was significantly different ($P = <0.0001$) but interaction of treatment and day was not significantly different ($P = 0.158$). The difference of the corneal lesion height between the treatment groups was not found to significantly different using a 95% confidence interval ($P = 0.108$) but the difference between days was found to be significantly different ($P = <0.0001$) but the interaction of treatment and day was not significantly different ($P = 0.244$). There was no appreciable difference in the amount of Na & CL in the plasma & serum samples among all three groups at any of the sampling time points. There was no difference between pre (Day 0) and post treatment (Day 11 and 17) in chlorine within Group 1 in the muscle, liver, fat & urine samples at any of the time points sampled in any of the calves sampled. The total protein of all calves utilized in the study was found to be within the normal reference ranges.

Discussion:

Management of IBK in cattle has been problematic due to many of the predisposing factors are often hard to control such as ultraviolet radiation, plant awns in grazing situations and fly control. Vaccinations have primarily focused upon the use of surface pili or cytolysin to stimulate host immunity.^{13, 25} *M. bovis* possesses a great potential for antigenic diversity and epitope conversion leading to a large variation in

antibody binding sites.²⁵ The development of efficacious vaccines relies on continued surveillance of new isolates recovered from outbreaks and the ability of the vaccine to have adequate antigen presentation.²⁵ A recent study assessed the ability of a *Moraxella bovis* pilin-cytotoxin-*Moraxella bovoculi* cytotoxin subunit vaccine to prevent naturally occurring infectious bovine keratoconjunctivitis.⁷ The vaccine was found not to be effective at preventing naturally occurring IBK and further research and development is needed in this area.⁷

Due to the problems with reliably immunity following vaccination utilization of antibiotic therapy is most often employed. Currently, the most common approved IBK treatments in the United States constitute parenteral administration of long-acting antibiotics, oxytetracycline (two injections of 20 mg/kg, IM or SC, at a 48- to 72-hr interval) and tulathromycin (2.5 mg/kg, SC, given once) each requiring meat withdrawals from 28 to 18 days, respectively.^{20,24} Oxytetracycline can be utilized in lactating dairy cattle with a milk withdrawal of 96 hours. However, tulathromycin is not labeled for use in lactating dairy cattle and it is not to be used in calves to be processed for veal due to lack of determination of a pre-slaughter withdrawal times for pre-ruminating calves. Also, tulathromycin effects on reproductive performance, pregnancy and lactation have not been fully determined. *Moraxella bovis* does not discriminate between dairy and beef cattle so IBK is problematic to the cattle industry as a whole. There are a number of other antimicrobials which have been utilized with some success for treatment of IBK causes by *M. bovis* and *M. bovoculi* including; penicillin, sulfadiazine, cephalexins, florfenicol, enrofloxacin, as well as myriad of topical preparations such as: triple antibiotic, gentamicin, and oxytetracycline ointments.^{7,17,20,24} Surgical intervention is sometimes utilized in severe cases, such as a third-eyelid flap or partial tarsorrhaphy. These surgical procedures are protective and shade the cornea from sunlight and prevent further contamination of the eye. Eye patches function in much the same way and may aid in preventing further transmission of the bacteria from one animal to the next.

Antibiotics have truly been the standard of care for *M. bovis* infections. However, it is an imperative to look at novel therapies that can decrease the need for antimicrobial treatment. The Food and Drug Administration (FDA) has emphasized this need in their document, “*The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals*”.³⁷ Concern for the ever growing antimicrobial resistance, and the resulting failure of antimicrobial therapies in humans has become a public health problem of global significance. This phenomenon is driven by many factors including the use of antimicrobial drugs in both humans and animals. The fear of loss of effectiveness and the documented loss of effectiveness of antimicrobial drugs has resulted in implementation of stricter regulations when utilizing drugs in food producing animals. The FDA along with the Centers for Disease Control (CDC), and the American Veterinary Medical Association (AVMA) have taken steps to help implement judicious use of antimicrobials in animals.^{1,3} Enactment of the Preservation of Antibiotics for Medical Treatment Act (PAMTA), seeks to eliminate the “nontherapeutic” use of antibiotic drugs considered to be important for human health in order to preserve the effectiveness of these antibiotics by decreasing the potential for development of antibiotic-resistant bacteria in humans.²⁹ Additionally, the World Health Organization (WHO) completed a strategic action plan on antibiotic resistance in 2011 and has many reports such as the one by Nordberg et al., 2005.^{18,26} These reports analyze antibiotic resistance and emphasize the need for prudent use of antimicrobials in humans and animals alike.^{18,26} The focus of these reports and emphasis has been primarily on feeding antimicrobials to food animals and parenteral administration of specific antibiotics known to stimulate bacterial resistance genes. Topical therapies are a growing concern as well. Therefore, there is significant pressure on the development of new therapeutic applications that do not promote antimicrobial resistance.

One novel therapy investigated the utilization of *Bdellovibrio bacteriovorus* 109J as an alternative non-chemotherapeutic and non-antibiotic treatment of IBK.¹² It was found that treatment with a *B. bacteriovorus* suspension did decrease adherence of *M. bovis* to cells in culture and the number of *M. bovis* in culture.¹² An additional alternative therapy, Vetericyn Plus™ Pink Eye Spray (Innovacyn, Rialto,

CA) has also been found to be even more effective in culture trials, reporting a kill 99.9% of *M. bovis* in product trials. However, further clinical trials were needed to more fully evaluate the efficacy of Vetericyn Plus™ Pink Eye Spray, hence, the study that has been presented herein. The results of this study indicate that this product reduces pain and infection in calves with experimentally induced IBK. The mechanism behind the reduction of pain and infection might be most likely through the action of hypochlorous acid, the active ingredient, and its sodium salt, sodium hypochlorite. Hypochlorous acid is considered to be the primary antimicrobial product of the myeloperoxidase (MPO) granules (azurophilic granules) of the neutrophil.² The MPO granules are critical to the oxidative burst.² Specifically, during the activation of neutrophils, respiratory bursts generate hydrogen peroxide which when acted upon by MPO produces hypochlorous acid.³³ Hypochlorous acid is highly active against all bacterial, viral, and fungal pathogens and can even kill spore-forming and non-spore bacteria in a short time period.³³

Hypochlorous acid acts to disrupt the biofilm that *M. bovis* produces as documented in a recent study.^{30, 33} Biofilm formation is now recognized as a serious problem in chronic wound infections.³³ Biofilms are a complex structure of microorganisms that generate a protective shell, allowing bacteria to collect and proliferate.³³ The biofilm structure of microorganisms renders phagocytosis difficult, and is known to increase resistance to antibiotics.^{30, 33} Hence, the ability of hypochlorous acid (Vetericyn Plus™ Pink Eye Spray (Innovacyn, Rialto, CA) to disrupt the biofilm of *M. bovis* results in prevention of further growth and corneal damage due *M. bovis*.

Elimination and or reduction of *M. bovis* alone will reduce pain since *M. bovis* releases hemolysin.¹¹ Hemolysin is known to result in cell lysis.¹¹ Additional factors such as other cytokines and proteolytic enzymes produced by the bacterium cause damage to the cornea.¹¹ However, *M. bovis* hemolysin is the major bacterial exotoxin implicated in the pathogenicity of *M. bovis*, as it has been found that non-hemolytic strains do not initiate disease.¹¹ Products that reduce the number of *M. bovis* organisms should logically decrease the amount of hemolysin, along with additional cytokines and proteolytic enzymes elaborated on the cornea. Hence, the reduction in corneal damage and pain. The action of spraying of Vetericyn Plus™ Pink Eye Spray (Innovacyn, Rialto, CA) alone helped to mechanically cleanse the eyes and remove the organism. The mechanical action of spraying was controlled for by spraying the same amount of 0.9% sterile saline in Group 2 eyes with the same bottle type and trigger as was used to spray Vetericyn Plus™ Pink Eye Spray (Innovacyn, Rialto, CA).

Wound healing is a complex and requires removal of infection, granulation and reconstruction of the tissue. It is said to have three overlapping continuous sequences of inflammatory, proliferative, and maturation.³³ Fibroblasts, are considered the primary synthetic cells in the repair process of most structural proteins used during tissue reconstruction.³³ Keratinocytes are also essential in wound healing and modulate fibroblast proliferation.³³ In a 2014 study by Sakaryra et al., it was found that hypochlorous acid had highly favorable effects on fibroblast and keratinocyte migration when compared to other antiseptics and increased wound healing.³³ The findings in the study herein reiterated this recent study in that corneal healing was increased in Group 1 (Vetericyn Plus™ Pink Eye Spray) in comparison to Group 2 (0.9% saline control).

The residue portion of this study was included to help determine if following ten days of ocular topical administration of Vetericyn Plus™ Pink Eye Spray would result in any detectable tissue residues. Since, Vetericyn Plus™ Pink Eye Spray's active ingredient is hypochlorous acid and sodium hypochlorite is an additional ingredient the DPD Colorimetric method was chosen to evaluate total chlorine levels before and after ten days of therapy with Vetericyn Plus™ Pink Eye Spray in urine, liver, fat, and muscle. Serum and plasma samples were also evaluated before and after 10 days of therapy. There were no detectable differences in chloride and sodium levels following evaluation by the AUCVM Clinical Pathology Lab. There were no differences in chlorine in any of the samples of urine, fat, liver, and muscle in Group 1 using the DPD Colorimetric method. Hence, there should be no residue concerns when utilizing Vetericyn Plus™ Pink Eye Spray in calves.

In conclusion, Vetericyn Plus™ Pink Eye Spray has a powerful and rapid killing effect on *M. bovis* in clinical trials and aids in reduction of pain, and decreases healing times following experimental induction of IBK corneal lesions. Early detection and application of therapy is most often the key in having a good prognosis with diseases and with IBK in cattle this is certainly the case. Early application of Vetericyn Plus™ Pink Eye Spray will have the greatest chance to reduce the clinical signs associated with *M. bovis* induced IBK. However, further economic evaluation of Vetericyn Plus™ Pink Eye Spray (Innovacyn, Rialto, CA) therapy versus that of parenteral administration of approved antibiotics such as tulathromycin, and oxytetracycline, is necessary to determine the economic advantages that the producer might reap with its use.

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