PEER REVIEWED

Preliminary evaluation of hypochlorous acid spray for treatment of experimentally induced infectious bovine keratoconjunctivitis

Julie Gard,[1] DVM, PhD, DACT; **Debra Taylor**,[1] DVM, MS, DACVIM; **Rachel Maloney**,[1] BS; **Megan Schnuelle**,[1] BS; **Sue Duran**,[1] RPH, MS, PhD, DICVP; **Phillip Moore**,[1] DVM, DACVO; **Will Justus**,[1] BS; **Paul Walz**,[2] DVM, MS, PhD, DACVIM; **Ricardo Stockle**,[1] DVM, MS, DAVBP; **Soren Rodning**,[3] DVM, MS, DACT; **Fred DeGraves**,[4] DVM, PhD; **Edzard van Santen**,[5] PhD; **Misty Edmondson**,[1] DVM, MS, DACT; **Annette M. O'Conner**,[6] BVSc, MVSc, DVSc, FAZCVs
[1]Department of Clinical Sciences, College of Veterinary Medicine, Auburn University, Auburn, AL 36849
[2]Department of Pathobiology, College of Veterinary Medicine, Auburn University, Auburn, AL 36849
[3]Department of Agriculture, Auburn University, Auburn, AL 36849
[4]Department of Animal Sciences, Western Kentucky University, Bowling Green, KY 42101
[5]Department of Crop, Soil, and Environmental Sciences, Auburn University, Auburn, AL 36849
[6]Department of Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA 50011
Corresponding author: Dr. Julie Gard, waldrja@auburn.edu

Abstract

Thirty dairy bull calves with normal ophthalmic examinations and culture-negative for *Moraxella bovis* were randomly assigned to 3 groups for a single eye block, randomized, blinded challenge study. Calves were housed in an accredited isolation facility. In calves assigned to Groups 1 and 2, a 0.6 cm corneal lesion was made on the left central cornea utilizing n-heptanol, and immediately inoculated topically with 1.0 x 10^7 of *M. bovis* (strain Epp63-300; origin: NADC). The left eye of calves in Group 3 was inoculated topically with *M. bovis*, but nothing further. In Group 1, 2 mL of 0.009% hypochlorous acid (Vetericyn Plus™ Pinkeye Spray) was sprayed topically onto each calf's cornea twice daily for 10 days. In Group 2, 2 mL of 0.9% saline was administered topically to each calf's cornea twice daily for 10 days. Each animal was scored for ocular pain twice daily. Samples from all eyes were collected for culture on days -7, 0, 1, 4, and 10. Eyes of all calves were stained daily with fluorescein, and digital photographs were taken of the lesion to assess healing of the cornea. Additionally, serum and plasma samples were collected from all calves on days 0, 1, 10, 11, and 17, and changes in sodium and chloride levels were evaluated. 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 N,N-diethyl-p-phenylenediamine (DPD) colorimetric method. Only calves in Group 2 were culture-positive for *M. bovis* during the study period. The average pain score and days to cure for Group 1 was lower than Group 2 ($P < 0.05$ and $P < 0.02$, respectively). Corneal lesion circumference of calves in Groups 1 and Group 2 differed ($P < 0.04$), and the difference between days was significantly different ($P \leq 0.0001$). There was no difference in Na or Cl levels in the plasma and serum samples among the 3 treatment groups at any sampling time points ($P \leq 0.0001$), and no difference between pre- (day 0) and post-treatment (day 11 and 17) in chlorine levels in muscle, liver, fat, and urine at any time point. Results of this study suggest that hypochlorous acid spray can be utilized as alternative therapy to reduce pain, infection, and healing time of corneal lesions in calves experimentally infected with *M. bovis*.

Key words: cattle, *Moraxella bovis,* pinkeye, hypochlorous acid, residues

Résumé

Trente veaux laitiers mâles, ayant une condition ophtalmique normale et une culture négative pour *Moraxella bovis,* ont été aléatoirement assignés à 3 groupes dans une étude dans un seul œil en blocs randomisés à l'insu. Les veaux étaient logés en paire selon leur groupe de traitement respectif dans un local d'isolement approuvé par l' AAALAC. Chez les veaux des Groupes 1 et 2, une lésion cornéenne de 0.6 cm a été pratiquée sur la cornée centrale gauche à l'aide de n-heptanol. À l'apparition de la lésion, 1.0 x 10^7 de *M. bovis* (souche Epp63-300; origine: NADC) ont été administrées par voie topique sur la cornée centrale gauche des veaux des Groupes 1 et 2. Les veaux du Groupe 3 ont uniquement reçu une administration topique cornéenne de *M. bovis* sur la cornée de l'œil gauche. Pour le Groupe 1, 2 mL de Vetericyn Plus™ Pinkeye Spray (VPPS) ont été administrés par voie topique sur la cornée de chaque veau deux fois par jour

pendant 10 jours. Pour le Groupe 2, 2 mL de saline ont été administrés par voie topique sur la cornée de chaque veau deux fois par jour pendant 10 jours. Un score de douleur a été attribué à chaque animal deux fois par jour. Des échantillons de chaque œil ont été collectés et mis en culture aux jours -7, 0, 1 à 5, et 10. Chaque jour, les yeux de tous les veaux ont été colorés avec la fluorescéine et des photographies digitales de la lésion ont été prises afin d'évaluer la guérison de la cornée. La dimension de chaque lésion était évaluée quotidiennement à l'aide du logiciel image J. De plus, des échantillons de sérum et de plasma ont été prélevés chez tous les veaux aux jours 0, 1, 10, 11, et 17, et les changements en sodium et chlorure ont été évalués. Des échantillons d'urine et des biopsies de foie, muscle, et gras ont été prélevés chez tous les veaux aux jours 0, 11, et 17 et évalués pour leur teneur en chlore à l'aide de la méthode colorimétrique DPD. Seuls les veaux du Groupe 2 ont été trouvés positifs en culture pour *M. bovis* durant la durée de l'étude. Le score moyen de douleur pour les Groupes 1 et 2 était de 0.310 +/- 0.252 et 1.43 +/- 0.510, respectivement, sur une échelle de 1 à 4 durant la période de traitement de 10 jours. Le nombre moyen de jours avant guérison a été de 2.25 +/- 1.49 et 5.875 +/1 2.20 jours pour les Groupes 1 et 2 (P = 0.0161), respectivement. Les circonférences des lésions cornéennes des veaux des Groupes 1 et 2 différaient (P = 0.0375), et la différence entre le nombre de jours était significativement différente (P = <0.0001). Il n'y avait pas de différence dans les niveaux de Na ou Cl dans les échantillons de plasma et de sérum parmi les 3 groupes de traitement à n'importe quel temps d'échantillonnage (P = <0.0001). Il n'y avait pas de différence entre le jour pré- (jour 0) et les jours post-traitement (jours 11 et 17) dans les niveaux de chlore du muscle, du foie, du gras, et de l'urine des veaux dans les groupes n'ont pas été différents à n'importe quel temps d'échantillonnage de l'étude. Les résultats de cette étude suggèrent que Vetericyn Plus™ Pinkeye Spray peut être utilisé comme thérapie alternative pour diminuer la douleur, l'infection, et le temps de guérison des lésions cornéennes chez les veaux expérimentalement infectés à *M. bovis*.

Introduction

Infectious bovine keratoconjunctivitis (IBK), commonly called pink eye, is a painful ocular condition caused by *Moraxella bovis* that affects beef and dairy cattle worldwide.[8,15] It is reportedly the most important ocular disease in cattle.[4,8,24,38] Other ocular diseases in cattle are often characterized by conjunctivitis, but rarely result in keratitis, and if keratitis is present it is minimal. There are a number of pathogens reportedly associated with IBK in cattle, such as bovine herpes virus-1 (BHV-1), the causative agent of infectious bovine rhinotracheitis (IBR). Clinical signs of the ocular form of IBR are severe conjunctivitis and edema of the cornea, which usually originates near the limbus, but corneal ulceration is not common.[8,19] *Mycoplasma* spp can cause conjunctivitis and keratitis, either alone or in conjunction with *M. bovis*.[8,38] Concurrent infection with pathogens like *Moraxella bovoculi, Mycoplasma* spp, and BHV-1 can increase the severity of IBK.[8,38] Estimated annual losses associated with decreased weight gain alone in affected cattle exceeds $150 million.[24,35]

Moraxella bovis is considered the primary causative agent of IBK in cattle.[5,21] In a study by O'Conner et al,[31] *M. bovoculi* was isolated from cattle with naturally occurring IBK;[31] others also reported *M. bovoculi* was isolated from ocular infections in calves, and reindeer as well.[4,12,31,38,42] Although, *M. bovoculi* is considered a risk factor for development of IBK,[6] a challenge study with *M. bovoculi* alone did not cause disease.[5,21] However, a challenge study with *M. bovoculi* + *M. bovis* or *M. bovis* alone did result in central corneal ulceration.[5,21] Currently 7 different serogroups of *M. bovis* are recognized.[12] Genetic predisposition along with plant awns, face flies, ultraviolet radiation, dry and dusty environmental conditions, and shipping stress are all risk factors associated with development of IBK in cattle.[15,39] In addition, nutritional risk, such as selenium and copper deficiencies, can contribute to the disease.[34,39] Infectious bovine keratoconjunctivitis is difficult to control in both confined feeding and range conditions, but appropriate management practices like fly control and reducing dust in feed can help reduce the incidence.[8] Vaccination has provided mixed results, but reportedly can reduce the severity of clinical signs.[5,7,8,10,11,12,16,19,29,35] Vaccination can be expensive, especially when efficacy of the vaccine is lacking. Historically, systemic antibiotic treatment of clinical cases has been the preferred method of treatment.[6,8,17,20,25]

The Centers for Disease Control, the Food and Drug Administration, the World Health Organization, and the American Veterinary Medical Association have encouraged the beef and dairy industries to reduce the use of antimicrobials.[1,3,18,30,40] Hence, further evaluation of alternative therapies for treatment of *M. bovis*-induced IBK in cattle is of paramount importance. Hypocholorous acid, the active ingredient in Vetericyn Plus™ Pink Eye Spray,[a] is the primary antimicrobial product of the myeloperoxidase (MPO) granules (azuorophilic granules) of neutrophils.[2] Therefore, the objective of this study was to evaluate the therapeutic efficacy of using hypochlorous acid spray (HAS)[a] for treatment of experimentally-induced IBK caused by *M. bovis* in cattle. It was hypothesized that this product would aid in inhibition and elimination of *M. bovis* from corneal lesions, improve corneal healing, and reduce pain following experimentally induced IBK. A second objective was to determine if topical use of hypocholorous acid spray to treat IBK would result in detectable tissue residues in serum, plasma, liver, fat, muscle, or urine.

Materials and Methods

Animal care and use criteria and research facility

Thirty dairy bull calves (8 Holsteins and 22 Jerseys) ranging from 2 weeks to 6 months of age were utilized in the study. The study protocol was approved by the Auburn

University Institutional Animal Care and Use Committee (IACUC). The number of animals utilized in the study were in accordance with IACUC standards requiring minimal animals be used in certain types of research studies, while still having enough statistical power to make treatment comparisons. The study was conducted from September 2014 to October 2014 in the Auburn University College of Veterinary Medicine Sugg Laboratory, an Association for Assessment and Accreditation of Laboratory Animal Care approved isolation facility. The environmentally controlled isolation facility is designed to keep ambient air temperature at 70 °F (21 °C) and 40% relative humidity, with each room having a separate air handler. Isolation rooms have a net animal holding area of 100 ft^2 (9.29 m^2), and an additional 50 ft^2 (4.65 m^2) of staging space outside the animal containment area. Each of the rooms is capable of providing BSL 2 containment for swine, cattle, poultry, and small ruminants. Each room has a separate heating/air conditioning unit and ventilating fan designed such that all air passes through the facility only 1 time (no re-circulated air); HEPA filters are installed over exit vents. The system is a 100%-air-through system (no recirculating air) that allows for a complete air exchange in each room every 4 minutes. The isolation facility was utilized to control for variables between the 2 trials, including time of year, temperature, ultraviolent light, wind, and weather, as well as exposure to wildlife and extraneous people.

Study overview

Two trials were conducted. Trial 1 utilized 18 calves with 6 per group, and Trial 2 utilized 12 calves with 4 per group. Trial 2 was performed immediately following cleaning and disinfection of the isolation rooms after the first trial was completed. The study was split into 2 trials because there were not sufficient rooms to accommodate all calves in the isolation facility at one time. The same personnel were involved in the conduct of both trials.

Two calves from Trial 1 and 2 calves from Trial 2 were removed at the completion of the study due to respiratory disease, and were not included in the statistical analysis of corneal healing. All calves in Trial 1 were included in assessment of chlorine residues in tissues, and chloride and sodium levels in serum and plasma.

A single-eye randomized, controlled challenge design was employed in accordance with previous study designs.[15,22] All animals were examined by a board-certified veterinary ophthalmologist. Calves were excluded from the study if there was evidence of ocular abnormalities. Thirty calves with normal ophthalmic examination and culture negative for *M. bovis* were utilized in the study, thus 10 calves/treatment. Calves were randomly assigned to 1 of 3 treatment groups.

- Group 1. A 0.6 cm corneal lesion was induced by instilling 98% n-heptanol[b] in the left eye of each calf in the group, followed by immediate topical inoculation with 1 x 10^7 *M. bovis* organisms[c] in the same eye.[12,14,21] The right eye served as a control; no corneal lesion was created nor was the eye inoculated with *M. bovis*. Beginning 24 hours after inoculation with *M. bovis*, the left and right eyes were treated topically with 0.009% HAS for a total of 2 mL/applications (3 sprays) twice daily for 10 days. The right eye was sprayed at the same dose, frequency, and duration as the left eye to determine if HAS would cause ocular abnormalities. All bottles of HAS were cultured prior to use to ensure no bacterial contamination was present. Nozzles of the spray bottles were cleaned with alcohol prior to each use,[d] and a separate spray bottle was used in each isolation stall.
- Group 2. A 0.6 cm corneal lesion was induced with 98% n-heptanol in the left eye of each calf in the group, followed by immediate topical inoculation with 1 x 10^7 *M. bovis* organisms in the eye.[c,12,14,21] Beginning 24 hours after inoculation with *M. bovis*, the left and right eyes were treated topically with 0.9% sterile saline[e] for a total of 2 mL/applications (3 sprays) twice daily for 10 days. The right eye served as a control; no corneal lesion was created, and the right eye was not inoculated with *M. bovis*. All bottles of sterile saline were cultured prior to use to ensure no bacterial contamination was present. The nozzles of the spray bottles were cleaned with isopropyl alcohol prior to each use, and the bottles and nozzles utilized were the same as those utilized in Group 1. A separate spray bottle was used in each isolation stall.
- Group 3. A corneal lesion was not induced in either eye of calves in this group. The left eye of each calf in Group 3 was inoculated with approximately 1 x 10^7 *M. bovis* organisms;[c] the right eye was not inoculated, thus serving as a control. Following inoculation, the left and right eyes were not treated.

The left and right eye of each calf was evaluated twice daily for evidence of pain and ocular discharge by individuals blinded to the treatment, and scored accordingly utilizing a scale of 1 to 4. Both eyes of each calf were stained daily with fluorescein stain[f] following pain scoring, and digital images were taken on days -4, and 0 through 10. Digital images of the lesions were analyzed utilizing image J.[g]

Samples were taken for bacterial culture at various time points throughout the study period. Prior to the study, a general physical examination, along with evaluation of serum total protein, was performed on each calf to determine if there was evidence of any underlying disease state, such as parasites or dehydration, which might inhibit corneal healing and result in bias. Serum and plasma samples were collected from all calves on days 0, 1, 10, 11, and 17 and evaluated for changes in sodium and chloride levels. Urine, 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.[h]

Pain scoring

The left and right eyes of each calf were evaluated twice daily for evidence of pain and discharge, and scored utilizing a scale of 1 to 4: 1 = eye pain normal (no blepharospasm, discharge, or tearing); 2 = mild pain (intermittent partial closure of the eye lid +/- ocular discharge and/or tearing); 3 = moderate pain (intermittent complete closure of the eyelid +/- ocular discharge and/or tearing); or 4 = severe pain (consistent complete closure of the eye lid +/- ocular discharge and/or tearing).

Housing and feeding

The calves were housed in paddocks at Auburn University and then placed in the Sugg Laboratory Isolation facility to acclimate for 4 days prior to the start of the study. The calves were housed in 100 ft^2 (9.29 m^2) rooms in isolation until day 11 of the study. Calves were kept in pairs according to the assigned treatments. All calves were fed an 18 to 22% protein grain diet twice daily for the duration of the study, with free choice hay and water, and milk and or milk replacer when applicable.

Ophthalmic examinations before trial enrollment

Ophthalmic examinations were performed by a board certified veterinary ophthalmologist. The initial ophthalmic examinations were performed on days -7 to -4 following sedation of each calf with xylazine[i] at 0.023 mg/lb (0.05mg/kg) IV. A sample for culture was taken from the cornea and conjunctiva of each eye of each calf utilizing an individual sterile culture-swab[j] for each eye. All swab samples were immediately taken to the Auburn University College of Veterinary Medicine (AUCVM) Bacteriology-Mycology Diagnostic Laboratory and cultured for *M. bovis* and other commensal bacteria. Only animals negative for *M. bovis* were utilized in the study. Further examination of each calf included a Schirmer tear test,[k,9] tonometry testing,[l,22,23,31] and fluorescein staining of the cornea.[f,24,36,41] The examination also included assessment of the adnexa, cornea, anterior chamber, iris, lens, and anterior vitreous through utilization of a trans illuminator[m] and slit-lamp biomicroscope,[m] and indirect and direct ophthalmoscopy was performed following pharmacologic pupillary dilation with tropicamide 1%[n] in accordance with procedures detailed previously.[24,36,41]

Inducing corneal ulcers

On day 0, calves were sedated with xylazine[i] (0.023 mg/lb; 0.05 mg/kg IV) and butorphanol tartrate.[o] The left cornea of each calf was anesthetized with topical 0.5% proparacaine[p] hydrochloride. The central corneal lesion was then created by utilizing n-heptanol.[b,13] Corneal scarification was accomplished by soaking a 0.6 cm diameter paper disc[q] in 98% n-heptanol for 1 minute.[13] The discs were then placed mid-cornea on the left eye for 2 minutes prior to removal. Both eyes were then stained with fluorescein[f] and photographed.[r] Corneal lesions were induced in the left eye of all calves in Groups 1 and 2, but not in Group 3; however, the eyes of calves in Group 3 were stained with fluorescein[f] and photographed.[r] A single dose of flunixin meglumine[s] (0.5 mg/lb; 1.1 mg/kg IV) was administered at the time of corneal lesion formation in calves in Groups 1 and 2, and prior to fluorescein staining of the cornea in Group 3 calves.

Corneal inoculation with Moraxella bovis

Moraxella bovis strain Epp63-300[c] was grown overnight on trypticase soy agar broth[t] (TSAB) supplemented with 10% sterile bovine blood at 98.6 °F (37.0 °C) in a 5% CO_2 atmosphere. During the morning of the challenge in each trial, a 0.65% saline suspension of *M. bovis* was prepared from a fresh isolate to obtain a density of 1 x 10^7 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 corneal lesion formation in calves in Groups 1 and 2 and ocular evaluation of calves in Group 3, the left eye of all calves were inoculated by pipetting the saline suspension containing 1 x 10^7 organisms of *M. bovis*[c] onto the left cornea. To confirm viability and a positive control, samples of inoculum were plated onto TSAB[t] prior to the challenge, and then again after inoculating the calves. For the negative control, samples collected with a sterile swab[j] were streaked out on TSAB.[t] New latex gloves were worn by research personnel when inoculating each calf in order to avoid cross contamination. Treatment of the eyes was delayed 24 hours after inoculation to allow *M. bovis* to colonize the scarified cornea, although previous studies report 8 hours to be sufficient time to allow for colonization.[14,21]

Bacterial cultures

Samples for bacterial culture were collected from each calf on days 0, 1, 4, 7, and 10. The eyes of calves in Group 3 and the right eye of calves in Groups 1 and 2 were cultured initially on days -7 to -4, and then on days 0, 1, 4, and 10.

Image J analysis

Digital images were taken following fluorescein staining from approximately 4 inches (10 cm) from the cornea of both eyes on study days -4 and 0 through 10. Specific evaluation and measurement of corneal lesions was completed through examination of the digital picture and utilization of Image-J software.[g] The Image-J program[g] contained a scale tool, which allowed each image to be scaled. This allowed for collection of accurate and comparable measurements suitable for statistical comparisons. The Image-J software[g] was used to determine the circumference, width, height, and area of all corneal lesions on a daily basis. Two calves in Group 1 and 2 calves in Group 2 were removed at the end of the study due to respiratory disease; they were not included in the statistical comparisons of corneal healing.

Total protein assessment

Between day -7 to -4, a 10 mL blood sample was collected from all calves via jugular venipuncture into the appro-

priate vacuum tubes,[u] then placed in a cooler and transported immediately to the AUCVM Clinical Pathology Laboratory for determination of total protein.

Sodium and chloride levels

On study days -4, 0, 1, 10, 11, and 17, serum and whole blood were collected from all calves in Group 1 as previously described. Samples were placed in a refrigerator or cooler and transported immediately to the AUCVM Clinical Pathology Laboratory for determination of sodium and chloride levels.

Biopsy procedures

On days 0, 11, and 17, urine, 2 liver biopsies, 2 muscle biopsies, and 2 fat biopsies were collected from all calves in Group 1. Samples were also collected to analyze for hypochlorous acid; chlorine residue was the targeted compound.

Liver biopsy procedure

Calves were shaved on the right mid-thorax at the level of the 10th intercostal space, and the site was aseptically prepared, then 2.5 mL of 2% lidocaine hydrochloride[v] was administered intradermal within the sterile site, and the site was scrubbed a second time. A #15 Bard-Parker® scalpel blade[w] was utilized to make a stab incision through the skin, then 2 liver biopsies were collected utilizing a Bard-Parker® 14-gauge biopsy needle.[w] A total of 20 to 25 mg of tissue (10 to 12 mg/biopsy) was collected in accordance with the procedure outlined by Herdt.[23] Samples were placed in labeled micro-centrifuge Eppendorf tubes,[x] then homogenized with 1 mL of sterile water following mincing of tissue with a 25-gauge needle[y] attached to a 3-mL syringe.[y] Samples were then vortexed[z] for 2 intervals of 60 seconds, with a 5-second rest between. Each sample was then sonicated[aa] twice at 60-second intervals with a 5-second rest between the intervals. The solution was then tested with the DPD colorimetric assay[h] for chlorine.

Muscle biopsy procedure

Two muscle biopsies were collected from each calf in Group 1. Biopsies were collected from the right mid-semimembranosus muscle following aseptic preparation and administration of 2.5 mL of lidocaine.[v] Samples were collected utilizing a Bard-Parker® 14 gauge biopsy needle,[w] placed in labeled micro-centrifuge Eppendorf tubes,[x] homogenized, sonicated, and assayed in the same fashion as the liver biopsy samples as described above.

Fat biopsy procedure

Two fat biopsies were collected from the fat pad at the base of the right ear from each calf in Group 1. Following aseptic preparation of the area, 0.5 mL of 2% lidocaine hydrochloride[v] was administered intradermally within the sterile site. A #15 mm Bard-Parker® scalpel blade[w] was utilized to make a stab incision through the skin in the anesthetized area. 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,[x] homogenized, sonicated, and assayed in the same fashion as the liver biopsy samples described above.

Urine collection procedure

Urine samples were collected free-catch into sterile urine specimen cups,[bb] 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 N,N-diethyl-p-phenylenediamine (DPD) colorimetric method.[h] This method utilized a Chlorine (free and total) Color Disc Test Kit, Model CN-66y[cc] and Chlorine Reagent Set, DPD Free and Total Chlorine.[h] Samples were run in duplicate. Each sample was run with a control in accordance with the directions provided with the test kit. The sample was placed in the sample tube provided, and then qs to 5 mL with sterile water; a control tube was also filled with 5 mL 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 to 3.4 mg/L, equivalent to 0 to 3.4 ppm. This test kit was utilized to check for chlorine residues (proxy for hypochlorous acid) in urine and blended liver with sterile water,[u] blended muscle tissue with sterile water,[u] and blended fat with sterile water.[u] If the readings were zero on day 11, the day 17 samples were not analyzed.

Statistical Analysis

Data were modelled using repeated measures analysis after evaluating residual plots for normality of data.[dd] Data were subjected to a natural log transformation to approximate a normal distribution. Correlated data were accounted for using the following linear model:[26-28] $Y = X\beta + Z\mu + e$ where Y was the vector of observations, X was the treatment design matrix (treatment with HAS or saline, sampling at day 1-10), β was the vector of fixed treatment effects, Z was the random effects design matrix (calf, block), μ was the vector of random block effects, and e was the vector of experimental error. To account for the non-independence of observations within calves, 5 correlation structures were tested (compound symmetric, first order autoregressive, Toeplitz, unstructured and variance components).[26-28] Models were compared using Akaike's information criterion.[26-28] Calf and block were included in models as random effects.[26-28] The Kenward–Roger correction was used for all models.[26-28] *P*-values ≤ 0.05 were considered significant.

Results

All calves in Groups 1 and 2 developed corneal ulceration in the left eye as determined by fluorescein staining. Clinical signs in the left eye were consistent with IBK, including ocular discharge, conjunctivitis, blepharospasm, and tearing. In an earlier unpublished study, clinical signs of IBK were not seen when corneal lesions were created and not inoculated with *M. bovis.* In the current study, there were no clinical signs of IBK in Group 3 calves, nor in the right eye of calves in Groups 1 and 2. Only the left eye of calves in Group 2 were culture-positive for *M. bovis* following initiation of treatment. Six of 8 calves in Group 2 were culture-positive for *M. bovis* at the end of the study (Table 1), while none of the calves in Groups 1 and 3 were *M. bovis*-positive at the end of the study. The abundant growth of environmental bacteria, specifically *Bacillus* spp, *Staphylococcus*, and *Streptococcus*, on the culture plates may have competitively inhibited *M. bovis*. After infection was established in the corneal lesions, *M. bovis* was able to compete with the environmental pathogens and was readily detected in culture. Additionally, the use of swabs to collect samples for culture may have reduced the likelihood of detecting *M. bovis* in cultures. Swabbing was chosen as the sampling method, even though it is not as reliable as corneal scraping and corneal washing; however, scraping and washing the cornea could have impaired healing of the corneal lesions.

The average pain score for calves in Group 1 was 0.31 +/- 0.25 over the 10 day period, and 1.43 +/- 0.51 for calves in Group 2 ($P \leq 0.05$). The average days to cure for calves in Group 1, defined as no measurable lesion based on fluorescein uptake, was 2.25 +/- 1.49 days compared to 5.88 +/- 2.20 days for calves in Group 2 ($P < 0.02$; Table 2). The corneal lesion circumference in Group 1 calves was significantly different ($P < 0.04$) than Group 2 calves, and there was a difference between sampling days ($P \leq 0.0001$; Table 3), but the interaction of treatment and day was not significant ($P = 0.33$). The measured corneal lesion width differed between the treatment groups ($P = 0.0147$), and the difference between sampling days was significant ($P \leq 0.0001$), but the interaction of treatment and day was not significant ($P = 0.329$). There tended ($P = 0.08$) to be a difference between

Table 1. Results of bacterial culture of the left eye of calves experimentally inoculated with *Moraxella bovis* and treated with hypochlorous acid spray* (Group 1), 0.9% saline spray (Group 2), or not treated (Group 3).

Group	Day 0	Day 1	Day 4	Day 10
1 - A	negative	negative	negative	negative
1 - B	negative	negative	negative	negative
1 - C	negative	negative	negative	negative
1 - D	negative	negative	negative	negative
1 - E	negative	negative	negative	negative
1 - F	negative	negative	negative	negative
1 - G	negative	Positive	negative	negative
1 - H	negative	negative	negative	negative
2 - A	negative	negative	negative	negative
2 - B	negative	negative	Positive	Positive
2 - C	negative	negative	Positive	Positive
2 - D	negative	negative	Positive	Positive
2 - E	negative	Positive	Positive	Positive
2 - F	negative	negative	Positive	Positive
2 - G	negative	negative	negative	negative
2 - H	negative	Positive	Positive	Positive
3 - A	negative	negative	negative	negative
3 - B	negative	negative	negative	negative
3 - C	negative	negative	negative	negative
3 - D	negative	negative	negative	negative
3 - E	negative	negative	negative	negative
3 - F	negative	negative	negative	negative
3 - G	negative	negative	negative	negative
3 - H	negative	negative	negative	negative
3 - I	negative	negative	negative	negative
3 - J	negative	negative	negative	negative

*Hypochlorous acid spray = Vetericyn Plus™ Pink Eye Spray, Innovacyn, Inc., Rialto, CA

Table 2. Days to cure for calves experimentally infected with *Moraxella bovis* and treated with hypochlorous acid spray* (Group 1) or 0.9% saline spray (Group 2).

Group and calf	Days to cure
1 - A	1.5
1 - B	4.5
1 - C	1.5
1 -D	1.5
1 - E	2.5
1 - F	1.5
1 - G	.5
1 - H	4.5
2 - A	3.5
2 - B	4.5
2 - C	10.5
2 - D	6.5
2 - E	4.5
2 - F	4.5
2 - G	6.5
2 - H	6.5

*Hypochlorous acid spray = Vetericyn Plus™ Pink Eye Spray, Innovacyn, Inc., Rialto, CA

Table 3. Percent change of the corneal lesion circumference in calves experimentally infected with *Moraxella bovis* and treated with hypochlorous acid spray* (Group 1) or 0.9% saline spray (Group 2).

Group & calf	Day 0-1	Day 0-2	Day 0-3	Day 0-4	Day 0-5	Day 0-6	Day 0-7	Day 0-8	Day 0-9	Day 0-10
1 - A	29.74	45.74	100	100	100	100	100	100	100	100
1 - B	13.97	36.92	53.58	62.93	100	100	100	100	100	100
1 - C	24.89	57.18	100	100	100	100	100	100	100	100
1 -D	43.00	51.39	100	100	100	100	100	100	100	100
1 - E	43.45	67.48	77.85	100	100	100	100	100	100	100
1 - F	36.47	70.36	100	100	100	100	100	100	100	100
1 - G	64.81	100	100	100	100	100	100	100	100	100
1 - H	49.58	55.35	61.42	69.56	74.15	100	100	100	100	100
2 - A	9.58	31.54	36.81	77.15	100	100	100	100	100	100
2 - B	26.94	52.38	53.77	62.03	81.04	100	100	100	100	100
2 - C	15.58	30.35	33.4	31.46	42.47	45.97	48.94	54.3	67.39	74.27
2 - D	9.65	30.68	29.53	40.43	46.18	62.55	78.93	100	100	100
2 - E	44.39	60.57	68.43	76.29	76.29	100	100	100	100	100
2 - F	48.26	67.08	78.35	78.84	93.14	100	100	100	100	100
2 - G	60.8	70.7	79.29	100	100	100	100	100	100	100
2 - H	37.04	49.86	51.7	65.42	69.44	73.09	75.77	100	100	100

*Hypochlorous acid spray = Vetericyn Plus™ Pink Eye Spray, Innovacyn, Inc., Rialto, CA

the measured corneal lesion areas of the treatment groups, and the difference of the lesion area between sampling days differed significantly ($P \leq 0.0001$; Table 4), but interaction of treatment and day was not significant ($P = 0.16$). The difference of the corneal lesion height between the treatment groups was not significant ($P = 0.11$), but the difference between sampling days was significant ($P \leq 0.0001$). The interaction of treatment and day was not significant ($P = 0.24$).

There was no appreciable difference in plasma and serum Na and Cl levels between the 3 treatment groups at any time, and no difference between pre- (day 0) and post-treatment (day 11) chlorine levels in Group 1 calves in muscle, liver, fat, or urine samples were detected at any time point. Total serum protein of calves utilized in the study was within the normal reference range.

Discussion

Management of IBK in cattle has been problematic due to multiple predisposing factors, such as ultraviolent radiation, foreign bodies, and fly control. Vaccines have been primarily directed towards developing immunity against surface pili or cytolysin to stimulate host immunity.[5,7,11,12,26] *M. bovis* possesses great potential for antigenic diversity and epitope conversion, leading to large variation in antibody binding sites.[12,15,29,39] Development of efficacious vaccines relies on continued surveillance of new isolates recovered from outbreaks, and effective antigen presentation.[29]

There are, however, problems with reliable immunity following vaccination, and as a result antibiotic therapy is often employed to treat clinical IBK.[8,17,20,25] Currently, the most common treatments approved for IBK in the United States include parenteral administration of long-acting antibiotics—oxytetracycline (2 injections at 9.1 mg/lb or 20 mg/kg SC at a 48- to 72-hr interval) and tulathromycin (1.13 mg/lb or 2.5 mg/kg, SC, given once).[20,25] Oxytetracycline can be utilized to treat IBK in lactating dairy cattle with a milk withdrawal of 96 hours; however, tulathromycin is not labeled for use in lactating dairy cattle, and is not approved for use in calves intended for veal due to a lack of an established pre-slaughter withdrawal time for pre-ruminating calves. A number of other antimicrobials have been utilized with some success for treatment of IBK, including penicillin, sulfadiamethoxine, cephalosporins, florfenicol, enrofloxacin, as well as myriad of topical preparations such as triple antibiotic, gentamicin, and oxytetracycline ointments.[7,8,17,20,25,29] These products, however, are not labeled for treatment of IBK, and extralabel use of fluroquinolones in food producing animals is prohibited, and extralabel use of gentamicin is highly discouraged because of the potential for prolonged tissue residues.

Although antibiotics have been the standard of care for *M. bovis* infections, it is imperative to explore novel therapies that can decrease the need for antimicrobial treatment. The Food and Drug Administration has emphasized this need in the document "*The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals*".[40] Concern over antimicrobial resistance, and the resulting failure of antimicrobial therapies in humans, has become a public health concern around the globe. The FDA, along with the Centers for Disease Control and Prevention and the American Veterinary

Table 4. Percentage change of the corneal lesion area in calves experimentally infected with *Moraxella bovis* and treated with hypochlorous acid spray* (Group 1) or 0.9% saline spray (Group 2).

Group & calf	Day 0-1	Day 0-2	Day 0-3	Day 0-4	Day 0-5	Day 0-6	Day 0-7	Day 0-8	Day 0-9	Day 0-10
1 - A	60.25	83.23	100	100	100	100	100	100	100	100
1 - B	16.91	45.8	65.2	75.18	100	100	100	100	100	100
1 - C	53.03	87.13	100	100	100	100	100	100	100	100
1 -D	12.05	82.84	100	100	100	100	100	100	100	100
1 - E	69.09	92.23	97.07	100	100	100	100	100	100	100
1 - F	41.04	84.7	100	100	100	100	100	100	100	100
1 - G	74.26	100	100	100	100	100	100	100	100	100
1 - H	53.57	55.1	67.93	80.68	86.34	100	100	100	100	100
2 - A	24.17	59.22	75.99	95.4	100	100	100	100	100	100
2 - B	37.55	73.32	78.68	88.64	96.57	100	100	100	100	100
2 - C	42.21	44.37	58.87	59.98	68.91	71.5	74.2	80.05	89.87	96.38
2 - D	19.61	48.58	49.04	68.43	72.64	83.31	93.97	100	100	100
2 - E	55.26	72.79	73.98	85.55	90.44	92.41	93.92	100	100	100
2 - F	66.82	84.3	93.77	94.41	99.41	100	100	100	100	100
2 - G	55.26	72.79	73.98	85.55	90.44	92.41	93.92	100	100	100
2 - H	43.73	66.68	73.07	82.41	87.71	91.34	93.39	96.06	98	99

*Hypochlorous acid spray = Vetericyn Plus™ Pink Eye Spray, Innovacyn, Inc., Rialto, CA

Medical Association, have taken steps to help implement judicious use of antimicrobials in animals.[1,3]

Alternative therapy with HAS can be used with or without concurrent antimicrobial therapy. It does not contribute to antibiotic resistance or drug residues, and can be economically beneficial to the producer. HAS was found to be very effective in culture, killing 99.9% of *M. bovis* in product development trials. However, additional clinical trials are needed to more fully evaluate the efficacy of HAS and to expand studies comparing it to antimicrobial therapy. Results of the present study suggest that HAS reduces pain and infection in calves with experimentally induced IBK. The mechanism behind the reduction of pain and infection is most likely through the action of the hypochlorous acid, the active ingredient, and its sodium salt, sodium hypochlorite. Hypochlorus acid is considered to be the primary antimicrobial product of the myeloperoxidase (MPO) granules (azuorophilic 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.[37] Hypochlorous acid is highly active against all bacterial, viral, and fungal pathogens, and can even kill spore-forming and non-spore forming bacteria in a short time period.[37]

Additionally, hypochlorus acid acts to disrupt the biofilm that *M. bovis* produces.[34,37] Biofilms are a complex structure of microorganisms that generate a protective shell, allowing bacteria to collect and proliferate, and are now recognized as a serious problem in chronic wound infections.[34] The biofilm structure of microorganisms makes phagocytosis difficult, and is known to increase resistance of bacteria to antibiotics.[34,37] Hence, treatment with HAS disrupts the biofilm produced by *M. bovis*, and prevents further growth of *M. bovis* and subsequent corneal damage.

Hemolysin is the major bacterial exotoxin implicated in the pathogenicity of *M. bovis*, as non-hemolytic strains do not cause disease.[11] Elimination and/or reduction of *M. bovis* alone will reduce pain since it releases hemolysin, which is known to cause cell lysis.[11] Additional factors, such as other cytokines and proteolytic enzymes produced by the bacterium, cause damage to the cornea.[11] HAS can kill *M. bovis,* thereby reducing and/or eliminating corneal damage and pain associated with hemolysin, cytokine, and proteolytic enzyme production. However, in a recent study by Di Girolamo and associates, cytokines were shown to have a beneficial effect, contrary to what was previously thought.[16] Specifically, cytokines interleukin-2 and interferon-α, when utilized as adjuvants in IBK bacterins, reduced eye injury caused by IBK and the number of diseased animals.[16] Thus, cytokines apparently can have differing effects on wound healing in calves with IBK, suggesting the need for more research on the role of cytokines in the pathogenesis of this disease.

In the current study, the use of HAS appeared to aid in prevention of fulminating *M. bovis* infection in calves when administered early in the disease process. No culture-positive calves were found in Group 1 following initiation of treatment with HAS. Previous studies by Gould et al, utilizing the same strain of *M. bovis* as in the present study, found that corneal infection was established in less than 8 hours following topical corneal inoculation.[21] In the study presented here, the post-

inoculation time was lengthened to 24 hours before treatment to allow sufficient time for infection to be established.

The controlled environment in the isolation facility eliminated a number of variables, such as temperature, ultraviolent light, wind, and weather, that could have confounded results of the study. The air circulated through the isolation room was filtered and maintained a constant temperature and humidity. However, the environment did create some concerns, particularly with regard to secondary bacterial infections and the effect on culture results because of the calves living in very close confinement. The lack of positive cultures in Group 1 calves was likely due to the HAS killing/inhibiting the *M. bovis* organisms, thus preventing additional colonization of the cornea. Calves in Group 2 served as a saline-treated control, and the majority of the calves in this treatment group were positive for *M. bovis* (Table 1).

Calves in Group 1 had fewer days to cure compared to Group 2. Wound healing is a complicated process and requires removal of infection, granulation, and reconstruction of tissue, and is said to have 3 overlapping sequences of inflammation, proliferation, and maturation.[37] Fibroblasts are considered primary synthetic cells found in the repair process of most structural proteins used during tissue reconstruction.[37] Keratinocytes are also essential in wound healing, and modulate fibroblast proliferation.[37] A 2014 study reported that hypochlorous acid had highly favorable effects on fibroblast and keratinocyte migration when compared to other antiseptics, and enhanced wound healing.[37] The positive effects of hypochlorous acid on fibroblasts and keratinocyte migration, along with killing and disruption of the *M. bovis* biofilm, may be the primary mechanisms behind decreased healing times and reduction in pain seen in calves treated with HAS compared to those treated with saline in the current study.

One of the objectives of the current study was to investigate if use of HSA caused detectable changes of total chlorine, chloride, and sodium in tissues and blood. The DPD Colorimetric method was chosen to evaluate total chlorine levels in urine, fat, muscle, and liver before and after 10 days of therapy with HSA. Additionally, blood levels of sodium and chloride were measured in serum and plasma before and after 10 days of treatment with HSA to determine if there were any alterations detectable in blood. Results indicated there were no changes in total chlorine in serum, plasma, urine, fat, muscle, and liver tissues following treatment with HSA. These findings support the Food Animal Residue Avoidance Databank's determination that a pre-harvest withdrawal time is not necessary following use of HSA.

Conclusions

Hypochlorous acid eye spray used to treat experimentally induced IBK had a rapid killing effect on *M. bovis*, aided in reduction of pain, and decreased healing times with no detectable residues or alteration in sodium or chloride in body tissues. Early detection and application of therapy is most often the key to having a good prognosis when treating diseases, including IBK. Further economic evaluation of topical HSA therapy vs parenteral administration of approved antibiotics, such as tulathromycin and oxytetracycline, is necessary to determine the full economic advantages that the producer might receive with its use.

Endnotes

[a]Vetericyn™ Pinkeye Spray, Innovacyn, Rialto, CA
[b]98% n-heptanol, Sigma-Aldrich, St. Louis, MO
[c]*Moraxella bovis*, strain Epp63-300; R. Rosenbusch laboratory; origin: NADC, Ames, IA
[d]BD Individually Foil Wrapped Alcohol Swabs, Becton Dickinson, Franklin Lakes, NJ
[e]0.9% Sterile Saline, Baster, Deerfield, IL
[f]Fluorescein Stain, Akorn, Lakeforest, IL
[g]National Institutes of Health, Rasband WS; http://imagej.en.softonic.com/
[h]DPD Colorimetric Method, Hach®, Loveland, CO
[i]Rompun™, Bayer, Shawnee Mission, KS
[j]BD CultureSwab™ EZ Collection and Transport System, Franklin Lakes, NJ; http://www.bd.com/ds/productCenter/CT-CultureSwab.asp
[k]Schirmer Tear Test, Merck Animal Health, Kenilworth, NJ
[l]Tono-pen XL, Medtronic Ophthalmics, Inc., Fridley, MN
[m]Trans Illuminator and Slit Lamp, Kowa Sl-15 biomicroscope, Kowa Ophthalmic Diagnostics, Torrance, CA
[n]Mydriacyl™, Bausch & Lomb Incorporated, Rochester, NY
[o]Torbugesic®, Zoetis, Kalamazoo, MI
[p]Proparacaine 0.5% Ophthalmic Solution, Bausch & Lomb Incorporated, Rochester, NY
[q]Kimwipes®, Kimberly Clarke, Lagrange, GA
[r]Nikon D7200 24.2 Megapixel Digital Camera, Nikon, Melville, NY
[s]Banamine®, Merck Animal Health, Madison, NJ
[t]Trypticase soy agar broth, Sigma-Aldrich, St. Louis, MO
[u]BD Vacutainer tubes, Becton Dickinson, Franklin Lakes, NJ
[v]2% Lidocaine Hydrochloride, Hospira, Lakeforest, IL
[w]Bard-Parker #15 Scapel Blade® and 14-gauge Bard-Parker Biopsy Needle, Aspen Surgical, Caledonia, MI
[x]Safe-Lock 1.5mL Micro-centrifuge tubes, Eppendorf, Hauppauge, NY
[y]3mL Luer Lock Syringe and 25-gauge 1-inch Needle, Becton Dickinson and Co., Franklin Lakes, NJ
[z]Fisher Scientific™ Mini Vortex Mixer, Thermo Scientific Inc, Waltham, MA
[aa]Virsonic 100 Sonicator, ViTis, Gardiner, NY
[bb]Sterile 4-oz Urine Specimen Cups, Thermo Scientific Inc, Waltham, MA
[cc]Chlorine (Free and Total) Color Disc Test Kit, Model Cn-66, Hach®, Loveland, CO
[dd]PROC MIXED, SAS 9.1, SAS Institute, Cary, NC

References

1. About Antimicrobial Resistance. Centers for Disease Control and Prevention. Available at: www.cdc.gov/drugresistance/about.html. Accessed September 18, 2016.
2. Amulic B, Cazalet C, Hayes GL, Metzler K, Zychlinsky A. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol* 2012; 30:459-489.
3. Antimicrobial Use and Antimicrobial Resistance. American Veterinary Medicine Association. Available at: www.avma.org/KB/Resources/Reference/Pages/Antimicrobial-Use-and-Antimicrobial-Resistance.aspx. Accessed September 18, 2016.
4. 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;57:789-795.
5. Angelos JA, Lane MV, Ball LM, Hess JF. Recombinant *Moraxella bovoculi* cytotoxin-ISCOM matrix adjuvanted vaccine to prevent naturally occurring infectious bovine keratoconjunctivitis. *Vet Res Commun* 2010; 34:229-239.
6. Angelos JA, Ball LM, Byrne BA. Minimum inhibitory concentrations of selected antimicrobial agents for *Moraxella bovoculi* associated with infectious bovine keratoconjunctivitis. *Vet Diagn Invest* 2011; 23:552-555.
7. 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; 73:1670-1675.
8. Angelos JA. Infectious bovine keratoconjunctivitis (pinkeye). *Vet Clin North Am Food Anim Pract* 2015; 31:61-79.
9. Beckwith-Cohen B, Elad D, Bdolah-Abram T, Ofri R. Comparison of tear pH in dogs, horses, and cattle. *Am J Vet Res* 2014; 75:494-499.
10. Billson FM, Hodgson JL, Egerton JR, Lepper AW, Michalski WP, Schwartzkoff CL, Lehrbach PR, Tennent JM. A haemolytic cell-free preparation of *Moraxella bovis* confers protection against infectious bovine keratoconjunctivitis. *FEMS Microbiol Lett* 1994; 124:69-73.
11. Billson, FM, Harbour C, Michalski WP, Tennent JM, Egerton JR, Hodgson JL. Characterization of hemolysin of *Moraxella bovis* using a hemolysis-neutralizing monoclonal antibody. *Infect Immun* 2000; 68:3469-3473.
12. Burns MJ, O'Connor AM. Assessment of methodological quality and sources of variation in the magnitude of vaccine efficacy: a systematic review of studies from 1960 to 2005 reporting immunization with *Moraxella bovis* vaccines in young cattle. *Vaccine* 2008; 26:144-152.
13. Cintron C, Hassinger L, Kublin CL, Friend J. A simple method for the removal of rabbit corneal epithelium utilizing n-heptanol. *Ophthalmic Research* 1978; 11:90-96.
14. Dewell RD, Millman ST, Gould SA, Tofflemire KL, Whitley RD, Parsons RL, Rowe EW, Liu F, Wang C, O'Conner AM. Evaluating approaches to measuring ocular pain in bovine calves with corneal scarification and infectious bovine keratoconjunctivitis-associated corneal ulcerations. *J Anim Sci* 2014; 92:1161-1172.
15. Dickey AM, Loy JD, Bono JL, Smith TPL, Apley MD, Lubbers BV, DeDonder KD, Capik SF, Larson RL, White BJ, Blom J, Chitko-McKown CG, Clawson ML. Large genomic differences between isolates acquired from the eyes of cattle with infectious bovine keratoconjunctivitis versus the deep nasopharynx of asymptomatic cattle. *Vet Res* 2016; 47:31.
16. DiGirolamno FA, Sabatina DJ, Fasa RN, Echegoyen M, Vela M, Pereira CA, Maure P. Evaluation of cytokines as adjuvants of infectious bovine keratoconjunctivitis vaccines. *Vet Immunol Immunopathol* 2012; 145:563-566.
17. Dueger EL, George LW, Angelos JA, Tankersley NS, Luiz KM, Meyer JA, Portis ES, Lucas MJ. Efficacy of a long-acting formulation of ceftiofur crystalline-free acid for the treatment of naturally occurring infectious bovine keratoconjunctivitis. *Am J Vet Res* 2004; 65:1185–1188.
18. European strategic action plan on antibiotic resistance. World Health Organization. EUR/RC61/14, EUR/RC61/Conf.Doc./7. Available at: http://www.euro.who.int/__data/assets/pdf_file/0008/147734/wd14E_AntibioticResistance_111380.pdf. Accessed September 18, 2016.
19. Funk L, O'Connor AM, Maroney M, Engelken T, Cooper VL, Kinyon J, Plummer P. A randomized and blinded field trial to assess the efficacy of an autogenous vaccine to prevent naturally occurring infectious bovine keratoconjuncitivitis (IBK) in beef calves. *Vaccine* 2009; 27:4585–4590.
20. Gokce HI, Citil M, Genc O, Erdogan HM, Gunes V, Kankavi O. A comparison of the efficacy of florfenicol and oxytetracycline in the treatment of naturally occurring infectious bovine keratoconjunctivitis. *Ir Vet J* 2002; 55:573–576.
21. Gould S, Dewell R, Tofflemire K, Whitley RD, Millman ST, Opriessnig T, Rosenbusch R, Trujillo J, O'Connor AM. Randomized blinded challenge study to assess association between *Moraxella bovoculi* and infectious bovine keratoconjunctivitis in dairy calves. *Vet Micro* 2013; 164:108-115.
22. Gum G, Gelatt, N, Miller D, MacKay E. Intraocular pressure in normal dairy cattle. *Vet Ophthalmol* 1998; 1:159-161.
23. Herdt LH. Liver biopsy in cattle. Available at: http://www.dcpah.msu.edu/sections/nutrition/WEBCD.NUTR.REF.002.pdf. Accessed September 18, 2016.
24. Knoop KJ, Dennis WR, Hedges JR. Ophthalmologic procedures. In: Roberts JR, Hedges JR, eds. *Clinical procedures in emergency medicine.* 5th ed. Philadelphia, PA: Elsevier Saunders, 2009; 63.
25. Lane MV, George LW, Cleaver DM. Efficacy of tulathromycin for treatment of cattle with acute ocular *Moraxella bovis* infections. *J Am Vet Med Assoc* 2006; 229:557-561.
26. Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. *J Anim Sci* 1998; 76:1216-1231.
27. Littell RC, Pendergast J, Natarajan R. Modelling covariance structure in the analysis of repeated measures data. *Stat Med* 2000; 19: 1793-1819.
28. Littell RC, Milliken GA, Stroup WW, Wolfinger RD, Schabenberger O. *SAS® for mixed models.* 2nd ed. SAS Institute Inc., Cary, NC, 2006.
29. McConnel CS, House JK. Infectious bovine keratoconjunctivitis vaccine development. *Aust Vet J* 2005; 83:506-510.
30. Nordberg P, Monnet DL, Cars O. Priority medicines for Europe and the world, a public health approach to innovation: antimicrobial drug resistance. World Health Organization. *Emerg Infect Dis.* 2006;12. Available at: http://www.medscape.com/viewarticle/522899_10. Accessed September 18, 2016.
31. O'Connor AM, Shen HG, Wang C, Opriessnig T. Descriptive epidemiology of *Moraxella bovis*, *Moraxella bovoculi*, and *Moraxella ovis* in beef calves with naturally occurring infectious bovine keratoconjunctivitis (pinkeye). *Vet Microbiol* 2012; 155:374-380.
32. Passaglia CL, Guo X, Chen J, Troy JB. Tobno-Pen XL calibration curves for cats, cows, and sheep. *Vet Ophthalmol* 2004; 7:261-264.
33. Preservation of Antibiotics for Medical Treatment Act. H.R.1552 — 114th Congress (2015-2016). Available at: www.congress.gov/bill/114th-congress/house-bill/1552. Accessed September 18, 2016.
34. Prieto C, Serra DO, Martina P, Jacobs M, Bosch A, Yantorno OM. Evaluation of biofilm-forming capacity of *Moraxella bovis*, the primary causative agent of infectious bovine keratoconjunctivitis. *Vet Microbiol* 2013; 166:504-515. doi: 10.1016/j.vetmic.2013.06.003. Epub 19 Jun 2013.
35. Pugh GW, McDonald TJ, Kopecky KE, Kvasnicka WG. Infectious bovine keratoconjunctivitis: comparison of infection, signs of disease and weight gain in vaccinated versus non-vaccinated purebred Hereford heifer calves. *Can J Vet Res* 1986; 50:136-142.
36. Rockett J, Bosted S. *Veterinary clinical procedures in large animal practice.* 1st ed. Clifton Park, NY: Thomson Delmar Learning, 2007; 229-234.
37. Sakarya S, Gunay N, Karakulak M, Ozturk B, Ertugrul B. Hypochlorous acid: an ideal wound care agent with powerful microbicidal, antibiofilm, and wound healing potency. *Wounds* 2014; 26:342-350.
38. Schnee C, Heller M, Schubert E, Sachese K. Point prevalence of infection with *Mycoplasma bovoculi* and *Moraxella* spp in cattle at different stages of infectious bovine keratoconjunctivitis. *Vet J* 2015; 203:92-96.
39. Snowder GD, Vteck DV, Cundiff V, Bennett GL. Genetic and environmental factors associated with incidence of infectious bovine keratoconjunctivitis. *J Anim Sci* 2005; 83:507-518.
40. The judicious use of medically important antimicrobial drugs in food-producing animals. Docket No. FDA-2010-D-0094 (GFI #209). US Department of Health and Human Services, Food and Drug Administration, Center for Veterinary Medicine. Available at: www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM216936.pdf. *Fed Reg* 77: 2012:22328-22329. Accessed September 18, 2016.
41. Townsend W. Examination techniques and therapeutic regimens for the ruminant and camelid eye. *Vet Clin North Am Food Anim Pract* 2010; 26:437-458.
42. Tryland M, Neves CGD, Sunde M, Mørk T. Cervid herpesvirus 2, the primary agent in an outbreak of infectious keratoconjunctivitis in semi-domesticated reindeer. *J Clin Microbiol* 2009; 47:3707-3713.