Changes in bacterial and fungal ocular flora of clinically normal horses following experimental application of topical antimicrobial or antimicrobial-corticosteroid ophthalmic preparations

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Objective—To determine effects of topical antimicrobial and antimicrobial-corticosteroid preparations on the ocular flora of horses.

Animals—40 horses.

Procedure—One eye was treated 3 times daily for 2 weeks with one of the following ointments: 1) neomycin-bacitracin-polymyxin B, 2) 0.6% prednisolone-0.3% gentamicin, 3) neomycin-polymyxin B-0.05% dexamethasone, or 4) treated (artificial tears) control. Contralateral eyes of treated control eyes served as untreated control eyes. Corneal and conjunctival specimens for bacterial and fungal cultures were collected prior to initiation of treatment, after 1 and 2 weeks of treatment, and 2 weeks after concluding treatment. Changes in culture growth quantity scores of bacterial and fungal species were analyzed.

Results—The most common species before treatment were the following: gram-positive bacteria included *Streptomyces* spp (66%), *Staphylococcus* spp (46%), *Bacillus* spp (32%), and *Streptococcus* spp (32%); gram-negative bacteria included *Moraxella* spp (28%), *Escherichia coli* (24%), *Acinetobacter* spp (18%), and *Enterobacter* spp (14%); and fungi included *Aspergillus nidulans* (56%), *Cladosporium* spp (32%), and *Aspergillus fumigatus* (22%). In all groups, the percentage of positive bacterial culture results, growth quantity score of gram-positive bacteria, and number of bacterial species isolated decreased at week 1 and increased at week 2, whereas growth quantity score of gram-negative bacteria decreased throughout treatment. Differences were not significant among groups. Fungal growth quantity score decreased during treatment in all groups. Repopulation of bacterial and fungal species occurred.

Conclusions and Clinical Relevance—All interventions decreased the number of microorganisms. Repopulation of normal flora occurred during and after treatment. (*Am J Vet Res* 2005;66:800–811)

Infectious keratitis is a common, painful, vision-threatening, and economically important disease of the equine eye. For various reasons, the equine species appears to be predisposed to development of severe infectious keratitis. The injured equine cornea may be more likely to become infected after loss of the protective epithelial barrier as a result of the ubiquitous nature of bacteria and fungi in the outdoor or barn environment and poor efficacy of host immune responses to prevent infection.[1]

In cases of corneal infection, knowledge of normal ocular flora and how it is altered in diseased states permits the clinician to prescribe appropriate treatment.[2,3] Studies of normal ocular flora have been performed in horses and other species.[2,4,5] In horses, cattle, elephants, cats, dogs, and humans, the normal conjunctival flora is primarily gram-positive bacteria, whereas in eyes with extraocular disease, gram-negative bacteria are isolated with greater frequency.[6-14] Gram-positive bacteria commonly isolated from normal equine eyes are *Staphylococcus*, *Streptococcus*, *Corynebacterium*, and *Bacillus* spp. Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas*, *Moraxella*, *Acinetobacter*, and *Neisseria* spp are less frequently cultured from normal equine eyes.[2,4,6] Fungal organisms may also be transiently harbored in the equine conjunctival sac. Bacteria isolated from nondiseased eyes include *Cladosporium*, *Alternaria*, *Fusarium*, *Aspergillus*, and *Penicillium* spp.[2,5] Isolation of fungi is much more common in horses and ruminants than in cats and dogs.[5,8-10,15-17] In 1 study,[5] fungi were isolated from the normal conjunctiva of 95% of horses, 100% of cows, 22% of dogs, and 40% of cats.

The ocular surface is normally protected from microbial invasion by several mechanisms. These include an intact epithelium; the flushing action by the tear film; mechanical removal of organisms by blinking; antibacterial components of the tears such as lysozyme, beta-lysin, lactoferrin, and leukocytes; and ocular surface immunity.[18-20] Further, the normal ocular

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flora is thought to play a defensive role against pathogenic microbes by depriving them of nutrients, producing antimicrobial-like substances, and occupying space on the corneal and conjunctival epithelium.[2,21-23] However, under diseased conditions, the microbes composing the normal flora may become opportunistic pathogens and known pathogens such as gram-negative bacteria and fungi are more frequently isolated.[3,7,8,11-13] Risk factors for equine infectious keratitis include seasonal influences such as weather, temperature and humidity, and type of housing and use of topical corticosteroids and antimicrobials.[24-27]

The injudicious use of topical antimicrobial and antimicrobial-corticosteroid preparations has been implicated in potentiation of corneal infection.[14,24-37] In vitro, corticosteroids do not appear to directly enhance microbial growth or interfere with the mechanism of action of antimicrobials.[38,39] However, corticosteroids have been shown to reduce the efficacy of some antifungal agents and may interact directly with fungal organisms to block antifungal activity.[40] Corticosteroids may also concurrently increase the opportunity for infection to occur by delaying epithelialization, potentiating enzymatic degradation of the corneal stroma, and inhibiting phagocyte mobilization and function as well as vascularization and infiltration of fibroblasts into the cornea.[36,41-47] Therefore, for many reasons, treatment with topical corticosteroids may predispose to infection by pathogenic microorganisms. Supporting this theory, several investigators have found that topical corticosteroid application enhanced experimentally induced *Candida albicans* keratitis in rabbits.[36,40]

In humans, it is believed that chronic use of topical antimicrobials results in a shift from the normal predominantly gram-positive flora to gram-negative bacterial and fungal flora and may facilitate the emergence of resistant bacterial strains.[14,48] In horses, this theory is supported by the increased prevalence of gram-negative bacteria and fungi in horses with ulcerative keratitis. Approximately two thirds of the affected equine eyes in 1 study[13] had been treated chronically with topical antimicrobials, compared with normal untreated equine eyes from which predominantly gram-positive flora was isolated.[4,6] Additionally, resistance of bacteria to certain antimicrobials was increased for isolates obtained after antimicrobial treatment, compared with isolates obtained before the institution of treatment.[49] In 65% to 91% of cases of fungal keratitis, antimicrobials had been used prior to examination.[13,26,29] Further evidence is provided by studies of Moore et al in which 38%[49] and 62%[13] of eyes with fungal keratitis had been previously treated with topical corticosteroids, whereas others documented prior treatment in 22% to 64% of the cases of fungal keratitis.[26,27,29,50] Treatment with a combination of an antimicrobial and a corticosteroid may have an additive effect on potentiation of infection by simultaneously reducing the normal protective flora and suppressing the ocular inflammatory response.

Although reports[17,32,33,51,52] of mycotic keratoconjunctivitis in cats, dogs, and other species are limited, keratomycosis is common in horses. Incidence of equine fungal keratitis ranges from 4.8% to 39%, varying with season and region of the country.[3,13,26,29,53] Bacterial keratitis is more common, composing 66% of cases of ulcerative keratitis in 1 study.[13] In studies by Moore et al,[13,49] mixed bacterial and fungal infections accounted for 29% to 54% of the cases of equine infectious keratitis. Infectious keratitis in horses has significant economic implications, and despite aggressive appropriate medical management, surgical management, or both, the prognosis for salvage of vision and a cosmetic globe is guarded. In previous studies,[25-27,29] 5% to 56% of eyes with keratomycosis were enucleated because of lack of response to treatment.

Bacterial and fungal isolates in normal and diseased treated and untreated eyes have been surveyed.[2-5,13] In the clinical setting, chronic (≥ 2 weeks) antimicrobial treatment, corticosteroid treatment, or both are thought to alter the normal ocular flora and potentiate or predispose to fungal or bacterial corneal infections in horses. However, to our knowledge, no study has been performed to evaluate the changes in equine ocular flora after topical treatment with antimicrobial and antimicrobial-corticosteroid preparations. The purposes of the study reported here were to survey the normal ocular flora of 40 horses in central Ohio and to determine the effects of topical antimicrobial and antimicrobial-corticosteroid ophthalmic ointments on equine corneal and conjunctival bacterial and fungal flora. The hypothesis was that chronic use of topical antimicrobials or antimicrobial-corticosteroid preparations would do one or more of the following: 1) promote a shift in bacterial flora from gram-positive to gram-negative bacteria, 2) promote a shift from normal nonpathogenic flora to known or opportunistic pathogens, and 3) promote an increase in the prevalence of fungal organisms.

Materials and Methods

Animals—Forty horses (19 mares and 21 geldings) were selected at the Alice Finley Research Center of The Ohio State University. In order of decreasing frequency, the Thoroughbred, Standardbred, Quarter Horse, Trakehner, Arabian, Appaloosa, and warmblood crossbreeds were represented. Ages ranged from 2 to 21 years with a mean age of 9.8 years and a median age of 9.5 years. The study was performed late in the summer during a dry period, and the horses were housed in 1 of 3 fenced paddocks with a large hay manger in the center. From each paddock, horses had access to a barn with a manure pack base on the floor and a wall open to the outside. Horses were provided with free-choice hay and water, and grain was fed daily. Horses were deemed free of ocular diseases by complete ophthalmic examination including biomicroscopy and direct ophthalmoscopy.

The study was approved by The Ohio State University Institutional Animal Care and Use Committee and was performed during the months of August and September. The 40 horses were each randomly assigned to 1 of 4 treatment groups (10 horses/group), and the eye to be treated was randomly selected. Each eye was treated 3 times daily for 14 days with approximately a 0.25-inch ribbon of one of the following ophthalmic ointments: 1) **neomycin-bacitracin-polymyxin B (BNP)**,[a] 2) **prednisolone 0.6%-gentamicin 0.3% (Pred-G)**,[b] 3) **neomycin-polymyxin B-0.05% dexamethasone (NPD)**,[c] and 4) artificial tears (ie, treated control eyes).[d]

The contralateral eye of the treated control eyes served as the untreated control. Corneal and conjunctival specimens for bacterial and fungal cultures were collected immediately prior to initiation of treatment, after 1 and 2 weeks of treat-

ment, and 2 weeks following the discontinuation of treatment.

Specimen collection—When needed, horses were sedated with xylazine hydrochloride (0.3 to 0.5 mg/kg, IV). Specimens for bacterial and fungal cultures were collected in the morning prior to administration of ophthalmic ointments and after ophthalmic examination. Specimens were collected from the cornea and conjunctiva of each of the treated and control eyes. A commercially available rayon culture swab[e] was moistened with liquid Stuart transport medium and was gently applied to the central corneal surface and rolled up and down, with care taken not to contaminate the swab on the conjunctival or palpebral surfaces. The conjunctival specimen was collected similarly by inserting the swab into the inferior cul-de-sac between the eyelid and the nictitating membrane. The swab was rotated in the cul-de-sac while avoiding contact of the swab with the eyelid. Corneal and conjunctival specimens for bacterial and fungal cultures were taken to the microbiology laboratory immediately following the conclusion of collection of all specimens.

Culture procedure—Bacterial culture (blood agar and MacConkey agar) and fungal culture (Sabouraud dextrose agar with gentamicin [25 µg/mL]) were inoculated with swab specimens. The blood agar and MacConkey agar were incubated at 37°C and examined for growth at 24 and 48 hours. The technical staff of the microbiology laboratory of The Ohio State University Veterinary Teaching Hospital, by use of standard microbiological and biochemical techniques, performed identification of isolates. Bacteria were isolated and identified, and growth quantity score was estimated on the basis of the quadrant method of plate streaking in which the heaviest growth of bacteria occurs in the first quadrant of the plate streaked and progressively decreases in each subsequent quadrant. For statistical purposes, the scores were categorized as follows: a) no growth on the plate (score of 0), b) very few colonies obtained only from the subculture of brain heart infusion broth (score of 0.5), c) few colonies in the first quadrant of the plate (score of 1), d) light growth in the first 2 quadrants (score of 2), e) moderate growth in the first 3 quadrants (score of 3), and f) heavy growth in the first quadrant and decreasing growth quantity in all other quadrants of the plate (score of 4). This method of quantification was used because a measurable quantity of material from the ocular surface could not be collected with a culture swab.

Sabouraud dextrose agar was incubated at 25°C and examined daily for growth of fungal colonies for 21 days before culture results were considered negative. Identification of fungal isolates was done by standard transparent adhesive tape mount preparations stained with lactophenol cotton blue. When lack of sporulation precluded identification from Sabouraud dextrose agar, subcultures were made onto oatmeal agar. In such cases, the block culture method of fungal identification was used. The total number of colonies present on the culture media was used to quantify fungal species and fungal pathogens. Antifungal susceptibility was not addressed in this study.

Statistical analyses—A repeated-measures ANOVA was performed for comparison of the treated eyes versus the untreated and treated control eyes. Because of the number of tests performed, a value of $P \leq 0.01$ was considered significant. For significant treatment effects, a priori contrasts and graphic analyses were constructed. For before-and-after comparisons of nonparametric data, Wilcoxon rank sum tests were completed. A value of $P \leq 0.05$ was considered significant. Commercial software was used to complete all analyses.[f,g]

The following variables were analyzed for differences in corneal and conjunctival specimens individually over time: mean growth quantity score of gram-positive bacteria, mean growth quantity score of gram-negative bacteria, potential gram-negative bacterial pathogens, number of bacterial species isolated per specimen, gram-positive to gram-negative ratios, mean growth quantity score of fungal colonies, mean growth quantity score of fungal pathogen colonies, and total number of bacterial pathogens. Potential pathogens were determined by review of the literature describing bacterial and fungal isolates in diseased equine corneas.[3,6,13,19-26] The following bacterial organisms were considered to be potential pathogens: *Staphylococcus* (ie, *Staphylococcus aureus* and *Staphylococcus intermedius*), *Streptococcus* (ie, *Streptococcus zooepidemicus* and α-hemolytic *Streptococcus* spp), *Corynebacterium*, *Pseudomonas* (including *Pseudomonas aeruginosa*), *Enterobacter*, *Citrobacter*, *Acinetobacter*, *Actinobacillus*, and *Moraxella* spp and *E coli*. The following fungal organisms were considered to be potential pathogens: *Aspergillus*, *Fusarium*, *Candida*, *Alternaria*, *Penicillium*, *Scopulariopsis*, and *Geotrichum* spp; the Phycomycetes class (eg, *Mucor* and *Rhizopus* spp) were also considered potential pathogens of the ocular surface.

Initial analyses consisted of repeated-measures ANOVA. The analyses were performed both with and without inclusion of the data from week 4. This was done to search for a difference in findings by excluding the culture data obtained after treatment was discontinued. As a result of the large number of tests performed, the alpha used as the criterion for rejecting the null hypothesis was set at 0.01.

In the event of a significant treatment versus time interaction or a significant treatment effect, Dunnett tests were first performed to compare the treatment eyes versus the control eyes at baseline. These tests were performed to ensure that no differences existed between the groups of horses before treatments were applied. If differences at baseline were detected, then a new repeated-measures model was computed by use of the baseline measurement as a covariate. In the situation of a significant treatment versus time interaction, graphs were used to describe the detected changes in the effects of treatments over time. When a significant treatment effect was found, a priori contrasts were performed. A significant *P* value for a contrast indicated that the treatment had an effect at some time point. Because all treatments were combined in the initial repeated-measures ANOVA, the contrasts were used to find which treatment had an effect. All analyses performed were for treatment versus time and treatment versus each treated (artificial tears) and untreated control group individually.

Results

Percentage of positive bacterial culture results—Pretreatment bacterial culture results were positive for a mean of 78% of the corneal specimens and 90% of the conjunctival specimens from all treatment groups. When bacterial culture results from corneal and conjunctival specimens were combined, bacteria were isolated from 46 of 50 (92%) eyes and at least 80% of the eyes in each group had positive bacterial culture results. No significant variation was found over time in the percentage of eyes from which bacteria were isolated; however, the number of eyes for which positive bacterial culture results were obtained decreased after 1 week of treatment in all 5 groups.

A 20% to 50% decline in positive bacterial culture results from corneal and conjunctival specimens was observed at week 1 in all groups except for corneas of treated (artificial tears) control eyes **(Figures 1 and 2)**.

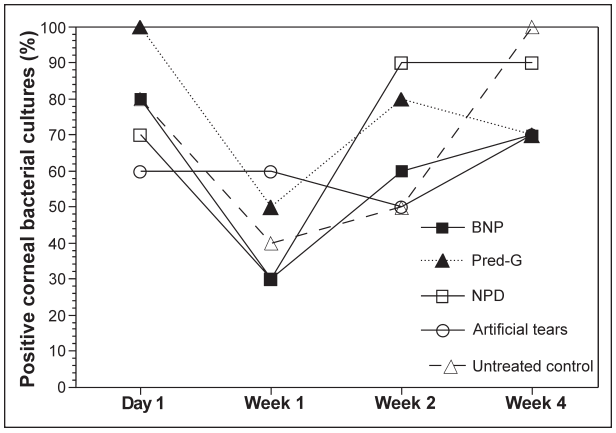

Figure 1—Percentage of positive corneal bacterial culture results for each treatment group and control eyes over time. BNP = Bacitracin-neomycin-polymyxin B. Pred-G = Prednisolone-gentamicin. NPD = Neomycin-polymyxin B-dexamethasone. Artificial tears = Treated control.

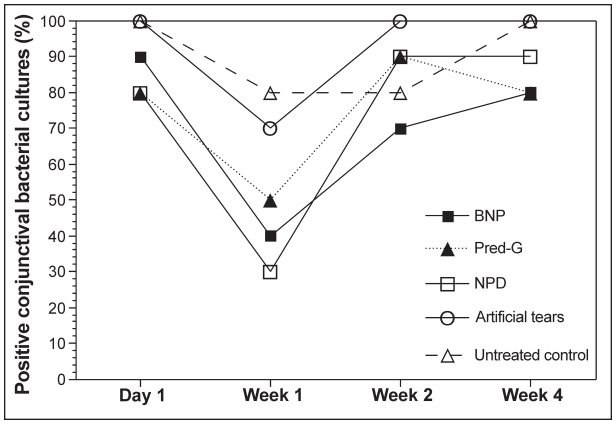

Figure 2—Percentage of positive conjunctival bacterial culture results for each treatment group and control eyes over time. *See* Figure 1 for key.

However, by week 2, the number of positive bacterial culture results from corneal and conjunctival specimens was higher than at week 1. No significant differences were found in the frequency of positive bacterial culture results from corneal and conjunctival specimens between treatment groups during the treatment period or between pretreatment values and those of specimens obtained during treatment (week 1 and week 2). However, at week 1, the percentage of positive bacterial culture results from conjunctival specimens was consistently lower for eyes treated with ointments containing antimicrobials (BNP, Pred-G, and NPD), compared with treated (artificial tears) and untreated control groups for which bacteria were isolated at the greatest frequency. In eyes treated with NPD, the percentage of positive bacterial culture results from corneal specimens increased significantly (P = 0.03) from week 1 to week 2 (30% to 90%, respectively) and from week 1 to week 4 (30% to 90%, respectively).

Descriptive analysis of bacterial species isolated—Culture results of corneal and conjunctival specimens were combined for evaluation of bacterial isolates for each eye at each time point. The percentage of eyes that had positive culture results for each isolate was evaluated, and no significant changes over time or between groups was found. A total of 20 bacterial species, 11 gram-positive and 9 gram-negative, were isolated (**Table 1**). Prior to treatment, gram-positive bacteria were cultured from 34 of 50 (68%) corneas, whereas gram-negative bacteria were cultured from 11 of 50 (22%) corneas. Compared with corneal specimens, conjunctival specimens had more positive bacterial culture results; gram-positive bacteria were isolated from 40 of 50 (80%) conjunctival specimens, and gram-negative bacteria were isolated from 24 of 50 (48%) conjunctival specimens. *Streptomyces* (66%), coagulase-negative *Staphylococcus* (46%), and *Bacillus* (32%) spp were the most common gram-positive isolates.

Gram-positive bacteria typically had low to moderate growth quantity scores (scores ≤ 2) throughout the study period (**Table 2**). Coagulase-negative *Staphylococcus* spp were commonly isolated at all time points in all groups including the antimicrobial (BNP) and antimicrobial-corticosteroid (Pred-G and NPD) preparation–treated groups. In groups treated with BNP and NPD, a reduction in isolation of coagulase-negative *Staphylococcus* spp was found at week 1 (2/10 and 4/10 positive culture results, respectively) and then the isolation frequency increased at weeks 2 and 4 (7/10 and 8/10 positive culture results, respectively). In the remaining groups (Pred-G and control eyes), an increasing number of coagulase-negative *Staphylococcus* isolates were found during and after the treatment period (weeks 1, 2, and 4).

The potentially pathogenic β-hemolytic *Streptococcus* spp were rarely isolated during the study period except in the treated (artificial tears) control group, from which a β-hemolytic *Streptococcus* sp was isolated from the same 2 horses at all time points. A β-hemolytic *Streptococcus* sp was also isolated from the untreated eye of 1 of these 2 horses at week 1. *Streptomyces* spp, isolated from 66% of all eyes prior to treatment (range for each treatment group, 50% to 80%), were isolated at a decreased frequency (0% to 30%) in all groups during and after the treatment period. *Bacillus* spp were isolated at an increasing frequency in all 5 groups from week 1 (30%) to week 2 (40% to 80%).

Moraxella spp (28%), *E coli* (24%), *Acinetobacter* spp (18%), and *Enterobacter* spp (14%) were the most common gram-negative isolates prior to treatment (Table 1). The remaining species were isolated from 2% to 8% of eyes before treatment. Gram-negative bacteria isolates were few in number and intermittent within treatment groups, and typically, gram-negative bacteria, except for *Moraxella* spp, had a low growth quantity score (ie, score of 0.5) at all time points (**Table 3**). *Moraxella* spp were eliminated in all eyes by week 2. At week 4, the number of positive culture results tripled, compared with the pretreatment number, in treated eyes but not control eyes. Nineteen of 30 (63%) treated eyes had positive culture results for *Moraxella* spp, compared with 4 of 20 (20%) control eyes (**Figure 3**). Additionally, at week 4, the growth quantity scores of *Moraxella* spp in treated eyes exceeded those of treated (artificial tears) and untreated control eyes: *Moraxella*

Table 1—Pretreatment gram-positive and gram-negative bacterial species isolated from all ocular specimens (corneal and conjunctival specimens combined) obtained from 40 horses.

Gram-positive bacteria	Positive culture results (%)	Gram-negative bacteria	Positive culture results (%)
Coag + *Staph*	6	*E coli*	24
Coag – *Staph*	46	*P aeruginosa*	2
α-hem *Strep*	18	*Pseudomonas* spp	6
β-hem *Strep*	4	*Enterobacter* spp	14
Nonhem *Strep*	14	*Citrobacter* spp	8
Corynebacterium spp	4	*Moraxella* spp	28
Enterococcus spp	2	*Acinetobacter* spp	18
Micrococcus spp	8	*Aeromonas* spp	0
Streptomyces spp	66	*Actinobacillus* spp	2
Bacillus spp	32		
Diphtheroid spp	10		

Coag + *Staph* = Coagulase-positive *Staphylococcus* spp. Coag – *Staph* = Coagulase-negative *Staphylococcus* spp. α-hem *Strep* = α-hemolytic *Streptococcus* spp. β-hem *Strep* = β-hemolytic *Streptococcus* spp. Nonhem *Strep* = Nonhemolytic *Streptococcus* spp. *E coli* = *Escherichia coli*. *P aeruginosa* = *Pseudomonas aeruginosa*.

Table 2—Percentage of eyes with positive culture results for each gram-positive bacterial species isolated (pretreatment, week 1, week 2, and week 4).

		Gram-positive bacteria (%)										
Treatment group	**No growth**	**Coag + *Staph***	**Coag - *Staph***	**α-hem *Strep***	**β-hem *Strep***	**Nonhem *Strep***	***Coryne***	***Enter***	***Microc***	***Strepto***	***Bacil***	***Diph***
BNP												
Pretreat	10	0	60	20	0	20	0	0	0	60	60	10
Week 1	30	10	20	0	0	0	0	0	0	20	30	10
Week 2	10	0	70	10	0	10	0	0	0	30	60	30
Week 4	10	0	70	10	0	20	0	0	20	0	60	30
Pred-G												
Pretreat	0	0	40	30	0	40	0	0	0	80	50	20
Week 1	20	10	60	20	0	20	0	0	0	20	30	30
Week 2	0	0	80	10	0	10	0	0	0	0	40	10
Week 4	10	0	70	10	10	0	0	0	20	10	50	50
NPD												
Pretreat	20	0	60	10	0	10	0	0	0	50	20	10
Week 1	40	0	40	0	0	0	0	10	20	30	30	30
Week 2	10	0	80	0	0	0	0	0	10	20	70	10
Week 4	0	0	80	40	0	10	0	0	0	10	40	20
Treated control												
Pretreat	0	10	30	20	20	0	10	0	20	70	10	0
Week 1	30	10	60	0	20	30	40	0	0	0	30	0
Week 2	0	0	80	30	20	20	0	0	0	0	80	30
Week 4	0	10	70	20	20	10	40	0	0	10	30	10
Untreated control												
Pretreat	0	20	40	10	0	0	10	10	20	70	20	10
Week 1	20	30	60	10	10	0	30	0	0	10	30	0
Week 2	20	20	80	20	0	0	0	0	0	10	40	20
Week 4	0	20	80	30	0	20	60	0	30	20	40	10

BNP = Bacitracin-neomycin-polymyxin B. Pred-G = Prednisolone-gentamicin. NPD = Neomycin-polymyxin B-dexamethasone. *Coryne* = *Corynebacterium* spp. *Enter* = *Enterococcus* spp. *Microc* = *Micrococcus* spp. *Strepto* = *Streptomyces* spp. *Bacil* = *Bacillus* spp. *Diph* = *Diphtheroid* spp.
See Table 1 for remainder of key.

spp had growth quantity scores ≥ 2.0 in 16 of 19 (84%) treated eyes but in only 1 of 4 control eyes.

Percentage of positive fungal cultures—Prior to treatment, fungi were isolated from a total of 26 of the 50 (52%) corneas and 39 of 50 (78%) conjunctival specimens. Regardless of treatment applied, the number of positive culture results was lower at week 1, week 2, and week 4, compared with before treatment (**Figure 4**). Significant differences were found within treatment groups between time points; however, in all groups, including the untreated control eyes, the changes occurred similarly. Therefore, the differences were not attributable to treatment effects.

Descriptive analysis of fungal species isolated—Corneal and conjunctival specimens were combined for evaluation of fungal isolates for each eye at each time point (**Table 4**). The most common species isolated prior to treatment were *Aspergillus nidulans* (56%), *Cladosporium* spp (32%), and *Aspergillus fumigatus* (22%). These species remained the most common at

Table 3—Percentage of eyes with positive culture results for each gram-negative bacterial species isolated (pretreatment, week 1, week 2, and week 4).

Treatment group	No growth	Gram-negative bacteria (%)								
		E coli	*P aeruginosa*	*Pseudomonas* spp	*Enterobacter* spp	*Citrobacter* spp	*Moraxella* spp	*Acinetobacter* spp	*Aeromonas* spp	*Actinobacillus* spp
BNP										—
Pretreat	10	10	0	10	10	0	20	10	0	0
Week 1	30	10	0	0	0	0	0	0	0	0
Week 2	10	20	0	0	10	10	0	0	0	0
Week 4	10	10	0	20	10	0	60	10	0	0
Pred-G										
Pretreat	0	10	0	0	0	10	20	10	0	0
Week 1	20	0	0	0	0	10	10	0	0	0
Week 2	0	40	0	0	0	0	0	20	0	0
Week 4	10	20	0	0	0	0	60	0	0	0
NPD										
Pretreat	20	30	10	0	30	10	40	20	0	0
Week 1	40	10	0	0	10	0	10	10	0	0
Week 2	10	10	0	0	0	0	0	0	0	0
Week 4	0	10	0	0	0	0	70	20	0	0
Treated control										
Pretreat	0	50	0	10	20	20	30	40	0	10
Week 1	30	20	0	0	20	0	10	40	0	0
Week 2	0	30	0	0	10	0	0	10	0	0
Week 4	0	10	0	30	0	0	30	50	0	0
Untreated control										
Pretreat	0	20	0	20	10	0	30	10	0	0
Week 1	20	10	0	10	0	0	10	20	0	0
Week 2	20	20	0	0	0	10	0	0	0	0
Week 4	0	30	0	20	10	0	10	50	10	0

See Table 1 for key.

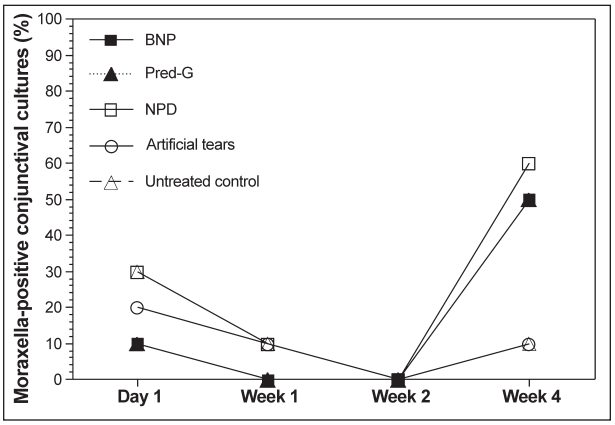

Figure 3—Percentage of conjunctival specimens with positive culture results for *Moraxella* spp for each treatment group and control eyes over time. *See* Figure 1 for key.

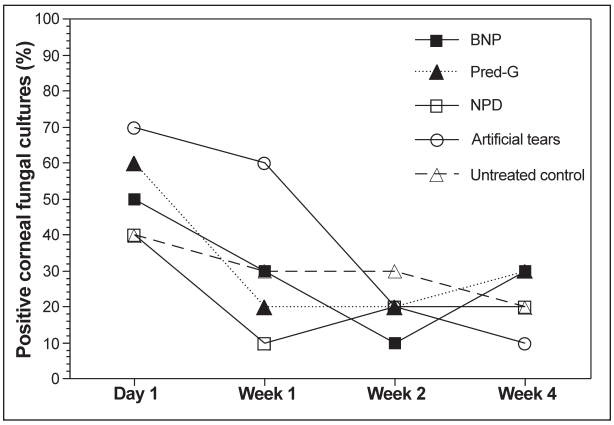

Figure 4—Percentage of positive corneal fungal culture results for each treatment group and control eyes over time. *See* Figure 1 for key.

weeks 1, 2, and 4, although the number of positive culture results for each species decreased to 25% to 75% of the baseline number as an overall decline in fungal populations occurred. The remaining fungal species were isolated infrequently throughout the study.

In light of the similar decline in growth quantity score of fungal colonies in all groups by weeks 2 and 4 (**Figure 5**), the significant (P = 0.004) difference in growth quantity score for conjunctival fungal pathogens in the NPD-treated group, compared with the control groups, does not appear to indicate a specific effect of treatment on fungal populations. No significant difference in growth quantity score was found for corneal fungal pathogens.

Quantitative analysis of bacterial and fungal cultures—No significant differences in number of bacterial species isolated per specimen were found for treated eyes, compared with control eyes. No significant effects of treatment over time were documented when treated eyes were compared with treated (artificial tears) or untreated control eyes for corneal or conjunctival mean growth quantity scores of gram-positive bacteria, gram-negative bacteria, potential gram-nega-

Table 4—Percentage of eyes in which each fungal species was isolated at each time point.

Fungal species	Positive fungal culture results (%)			
	Pretreatment	Week 1	Week 2	Week 4
A nidulans	56	54	32	26
A fumigatus	22	16	4	10
Cladosporium spp	32	8	8	12
Scopulariopsis spp	6	2	4	4
Alternaria spp	6	0	0	2
Stemphylium spp	4	8	2	0
Mucor spp	4	0	4	2
Fusarium sp	2	0	0	2
Curvularia sp	2	1	0	0
Scedosporium sp	2	0	0	0
Penicillium sp	2	0	0	0
Cephalosporium sp	2	0	0	0
Epicoccum sp	0	2	0	0

A nidulans = Aspergillus nidulans. A fumigatus = Aspergillus fumagatus.

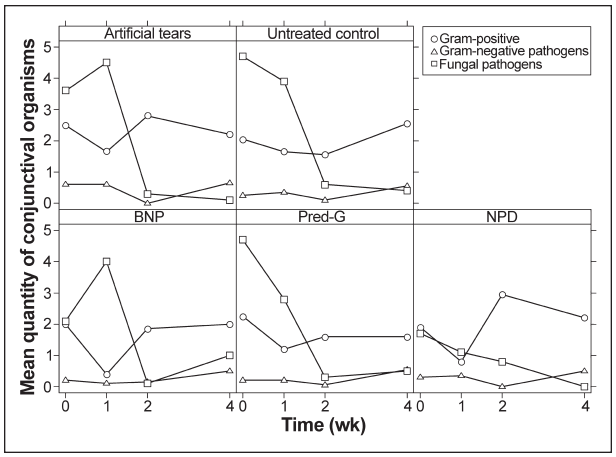

Figure 5—Trellis plot of gram-positive bacteria, gram-negative bacterial pathogens, and fungal pathogens isolated from conjunctival specimens. *See* Figure 1 for key.

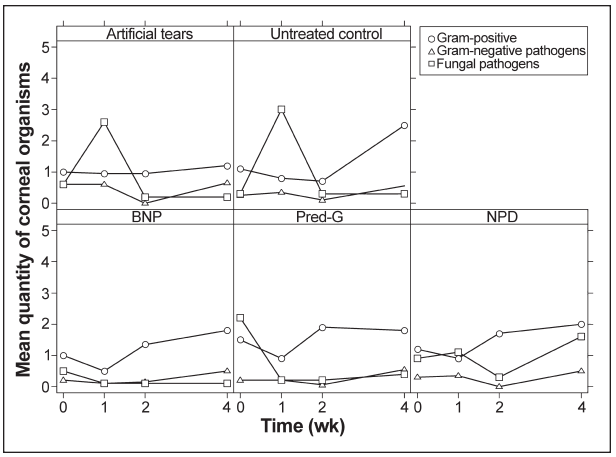

Figure 6—Trellis plot of gram-positive bacteria, gram-negative bacterial pathogens, and fungal pathogens isolated from corneal specimens. *See* Figure 1 for key.

tive bacterial pathogens, gram-positive to gram-negative ratios; mean growth quantity score of total bacterial and fungal pathogens; or mean growth quantity score of corneal gram-negative bacteria. However, for corneal and conjunctival specimens, significant differences between time points were found in treated eyes that were not attributable to treatment effects.

When week 4 data were excluded from statistical analyses to determine whether significant treatment effects were found during the treatment period, differences in conjunctival gram-positive to gram-negative bacterial ratios were found at certain time points for various treatments versus treated (artificial tears; P = 0.003) and untreated (P = 0.002) control groups. However, no consistent effects of treatment (ie, a treatment consistently increasing or decreasing the ratio) were identified.

Graphic analyses of interactions between bacteria and fungi—Graphic analyses were performed for conjunctival and corneal mean number of gram-positive bacteria, gram-negative bacteria, and fungal pathogens (Figures 5 and 6) to evaluate interactions between the microorganisms. For the mean growth quantity score of gram-positive bacteria, a decrease was found at week 1, and an increase occurred from week 1 to week 2 in all groups except the untreated control. There were minimal changes in growth quantity score of gram-positive bacteria in the untreated control during the study period. For gram-negative bacteria, a slight decline in growth quantity score was found by week 2 in all groups. The mean growth quantity score of fungal pathogens decreased to a low number in all groups at week 2 and remained low at week 4. Fungal growth quantity score varied inversely and proportionately to the growth quantity score of gram-positive bacteria at week 2 in all groups except the untreated control (ie, fungal growth quantity score decreased as gram-positive bacterial growth quantity score increased).

The growth quantity score of gram-positive bacteria decreased at week 1 and increased at weeks 2 and 4 in treated eyes but not in treated (artificial tears) or untreated control eyes (Figure 6). The growth quantity score of gram-negative bacteria changed little but decreased at week 2 and increased slightly at week 4 in all groups. Fungal pathogens increased in both control groups at week 1, were isolated in low numbers from

all 5 groups at week 2 as gram-positive bacteria repopulated, and remained low at week 4 while the growth quantity score of gram-positive bacteria remained constant or increased slightly.

Discussion

Habitation of the normal equine cornea and conjunctiva by bacteria and fungi is common. In our study, a mean of 78% corneal and 90% conjunctival bacterial cultures were positive for organisms prior to initiation of treatment, and when corneal and conjunctival specimens were combined, bacteria were isolated from 46 of 50 (92%) eyes. Fungi were isolated from a total of 26 of 50 (52%) of the corneal and 39 of 50 (78%) of the conjunctival specimens. These findings are similar to most prior surveys of culture results in normal equine eyes.[2,5,54] In 1 study,[4] however, bacteria and fungi were isolated from only 30% of eyes. The low frequency of microbial isolation in that study was attributed to seasonal effects, as the cultures were collected in the months of January and February, when the influence of microbial vectors such as dust, pollen, and face flies would be minimal. Our study was performed in a dry period during the late summer, when dust and face flies were at a maximum. These factors increased the likelihood of introduction of bacteria and fungi onto the ocular surface and thereby contributed to the high frequency of microbial isolation in the normal eyes before experimental manipulation.

The pretreatment bacterial flora of this equine population is comparable to that previously published for horses in other geographic regions[2,3,6,13,54-56] and therefore provided an appropriate estimate of normal equine ocular flora. Presence of gram-positive bacteria in corneal and conjunctival specimens (68% and 80%, respectively) exceeded that of gram-negative bacteria (22% and 48%, respectively); this fact is comparable to a prior study in which gram-positive bacteria represented 77% of ocular bacterial isolates and gram-negative organisms composed 23% of the isolates.[2] The most common gram-positive bacteria in our study were *Streptomyces* spp, coagulase-negative *Staphylococcus* spp, *Bacillus* spp, and α-hemolytic and nonhemolytic *Streptococcus* spp. The most common gram-negative bacteria were *Moraxella* spp, *E coli*, *Acinetobacter* spp, and *Enterobacter* spp. These gram-positive and gram-negative bacteria represent the normal flora as shown in previous surveys of clinically normal horses.[2,3,6,13,54-56] A low prevalence of potential pathogens, such as β-hemolytic *Streptococcus* spp, coagulase-positive *Staphylococcus* spp, and *Pseudomonas* spp, was found in our study. Additionally, when these species as well as other gram-negative bacteria such as *E coli* and *Acinetobacter* and *Enterobacter* spp were isolated, they had low growth quantity scores (ie, scores of 0.5). Because these organisms are ubiquitous in the environment, the low isolation frequency and growth quantity score of the pathogenic bacteria suggest that the normal flora and a healthy local environment may control or suppress colonization by pathogens.[2]

No significant detrimental effects of treatment on the normal ocular floral balance were found in our study. Rather, it was shown that the frequency of positive culture results for gram-positive bacteria decreased and that the growth quantity score of gram-positive bacteria declined after 1 week, then increased by week 2. Declines in gram-positive bacterial populations were more notable, as expected, in eyes that were treated, compared with untreated control eyes, particularly in corneal specimens, but a slight decline was also observed in the untreated control during the treatment period. The decline from week 1 to week 2 in susceptible gram-positive bacteria was expected with the application of the BNP, Pred-G, and NPD, as all of these preparations contained bactericidal antimicrobials. Several gram-positive bacteria were notably affected by treatment. The α-hemolytic *Streptococcus* spp and nonhemolytic *Streptococcus* spp were both eliminated at week 1 in groups treated with BNP and NPD, but not in eyes treated with Pred-G. This is compatible with known resistance of these species to gentamicin.[57] Coagulase-negative *Staphylococcus* spp were commonly isolated prior to treatment, were reduced at week 1, and were isolated at an increased frequency at weeks 2 and 4. Potentially pathogenic coagulase-positive and coagulase-negative *Staphylococcus* spp, including *S aureus* and *Staphylococcus epidermidis,* and *Streptococcus* spp, may have resistance to gentamicin, neomycin, and polymyxin B.[43,49] The isolation frequency of *Bacillus* spp also increased from week 1 to week 2 to contribute to bacterial repopulation of the ocular surface.

Repopulation at week 2 was primarily with the same species that were most prevalent prior to treatment. *Staphylococcus* spp and *Bacillus* spp have been isolated from normal and diseased equine eyes and are typically nonpathogenic except in situations of corneal compromise when opportunistic infection may occur.[2-4,6-13] *Streptomyces* spp, although found commonly in normal eyes,[2] have not been typically isolated from diseased eyes and do not appear to be likely pathogens.[23] *Streptomyces* spp, isolated from 66% of the specimens prior to treatment, were isolated at a decreased frequency in all groups during and after treatment.

Gram-negative bacteria did not repopulate to pretreatment amount until after treatment was discontinued (week 4). Again, repopulation occurred with the same organisms that were prominent prior to treatment (*Moraxella* spp, *E coli, Acinetobacter* spp). *Escherichia coli* and *Acinetobacter* spp have been isolated more frequently in diseased than normal equine eyes. In most studies, isolation of *Moraxella* spp has been infrequent in both normal (2% to 5%) and diseased (2.4% to 2.6%) eyes, but *Moraxella* spp were isolated from 86% of nondiseased eyes in 1 survey.[3,13,58] In a more recent survey in Florida, *Moraxella* spp were isolated at the highest frequency in the month of October (10.5%) and most commonly in young horses.[56] *Moraxella* spp are commonly known as pathogenic inhabitants of mucous membranes, the respiratory tract, and other sites in humans, and *Moraxella bovis* is a causative agent of infectious bovine keratoconjunctivitis.[20] A nonhemolytic *Moraxella* sp was isolated from eyes of ponies and a horse with naturally occurring conjunctivitis.[59] Other than that report and rare cases of *Moraxella* spp isolation in eyes with ulcerative

keratitis,[13] this organism appears to be a variably present nonpathogenic component of the normal equine flora. *Moraxella* spp were eliminated after 1 week in all treated eyes and were not isolated from any eyes, including control eyes, after 2 weeks of treatment. Thus *Moraxella* organisms appear to be susceptible to the antimicrobials used, but their presence may also be influenced by environmental factors.

It is not clear why repopulation would begin while antimicrobial treatment was being administered. There are several possible explanations for these findings. First, the low growth quantity score of bacteria and as few as 1 species, even a contaminant, resulted in a positive culture designation. Second, complete sterilization of the conjunctiva or cornea would be unlikely as a result of variation in susceptibility of organisms to the antimicrobials used,[13,49] propagation of resistant organisms as susceptible ones are suppressed by antimicrobial treatment,[49,60] lack of continual presence of antibacterial medication,[43,61] and constant microbial repopulation of the eye from organisms ubiquitous in the immediate environment. In normal eyes, resistance of gram-negative bacteria, particularly *Pseudomonas* spp, may be infrequent, but in previously treated diseased eyes in a study by Moore et al,[49] resistance to these antimicrobials was more prevalent. In previous susceptibility studies,[13,49] the antimicrobials used in our study were not 100% effective in eliminating all of the bacterial species isolated in our study.

It is also important to consider factors other than antimicrobial treatment that would affect the ocular bacterial populations. In a study[2] of microbial isolates in hospitalized versus stabled horses, no difference was found in bacterial isolates between the 2 populations. Therefore, prevalence of bacteria may be more dependent on host factors such as age, breed, season, diet, cutaneous flora, contact with other animals, and grooming than variation in local environmental factors.[2] Supporting this theory, a recent study[56] found a higher incidence of isolation of gram-negative bacteria, *Moraxella* spp, and fungal species from conjunctival specimens in young horses (< 5 years). The influence of age was not evaluated as a factor in our study.

Bacterial populations declined in treated (artificial tears) and untreated control eyes during the treatment period. Declines in the treated (artificial tears) control group could be attributed to removal of bacteria by mechanical flushing by the lubricant and reflex lacrimation or to antimicrobial effects of the preservative in the preparation. However, because untreated eyes also experienced a decline, an environmental change in the presence of bacteria or changes in the host may also be implicated.

In our study, known gram-positive pathogens such as *S aureus* and β-hemolytic *Streptococcus* spp were rarely isolated and were found to have low growth quantity scores (ie, scores of 0.5 to 1.0) throughout the study period. In fact, β-hemolytic *Streptococcus* spp were isolated only in the same 2 horses at every time point, further supporting the role of individual host factors in the balance of microbial flora. Except for *Moraxella* spp, gram-negative bacteria, particularly known pathogens such as *P aeruginosa*, had low growth quantity scores (ie, scores of 0.5 to 1.0). Pathogens have also been isolated in low frequency in other studies of normal equine flora.[2] Significant increases in the growth quantity score of gram-negative bacteria were not seen with chronic antimicrobial or antimicrobial-corticosteroid preparation treatment. On the contrary, growth quantity scores of gram-negative bacteria were significantly lower for eyes treated with BNP and Pred-G than for treated (artificial tears) control eyes. Repopulation occurred primarily with nonpathogenic *Moraxella* spp and gram-negative bacteria that had low growth quantity score prior to treatment, namely *E coli* and *Acinetobacter*.

Gram-positive to gram-negative ratios favored gram-positive bacteria prior to treatment for the BNP-treated, Pred-G–treated, and untreated eyes. By week 1, gram-positive bacteria exceeded gram-negative for all groups, and during the second week of treatment, an increase in the gram-positive to gram-negative ratios occurred in all groups. These results disprove the hypothesis that a shift towards gram-negative bacteria would occur in eyes chronically treated with antimicrobials or antimicrobial-corticosteroid preparations, as seen in clinical patients.[3,13] One study[49] that compared the gram-positive to gram-negative ratios in isolates from normal untreated eyes, initial isolates from ulcerated eyes, and isolates after antimicrobial treatment found a significantly greater frequency of gram-negative isolates in treated ulcerated eyes, compared with normal untreated eyes. The lack of effect of treatment to induce a shift towards gram-negative bacteria in our study may be multifactorial. First, in healthy eyes in our study, isolated gram-negative bacteria had a low growth quantity score. Therefore, it is possible that changes to the local ocular environment, such as an insult to the corneal epithelium and compromise of the normal floral balance (ie, elimination of normal flora), are required for gram-negative bacteria to proliferate to substantial numbers to cause clinical disease. Further, although repopulation with bacteria occurred during treatment, the bacteria were not capable of proliferating to any substantial degree without an ocular injury. Additionally, corticosteroids are thought to potentiate corneal infection by impairing epithelial regeneration, corneal vascularization, and inflammatory cell defenses against microbial invasion.[44-47] In a healthy eye, microbes are present in low numbers and no compromise is found of the local environment to induce the inflammatory changes that are impaired by corticosteroids. Therefore, the influence of topical corticosteroids on the microbial population in healthy eyes may be negligable.[37]

Fungal isolates obtained before treatment in our study were also typical of those in healthy equine eyes as described in previous reports.[2,5,55,56,62] *Aspergillus* spp were isolated from 78% of eyes and *Cladosporium* spp in 32% of eyes. Remaining isolates were isolated from only 2% to 6% of the eyes. In a composite of several studies of normal ocular flora, *Aspergillus*, *Penicillium*, and *Cladosporium* spp were the most common organisms isolated.[55] Interestingly, in that study, a common fungal pathogen, *Fusarium* sp, was not isolated from any of the healthy eyes, but was isolated from 1% to

32% of diseased equine eyes. In our study, *Fusarium* sp was cultured in only 1 eye. Additionally, *Penicillium* sp and *Cladosporium* sp, although common in healthy eyes, were infrequently cultured from diseased eyes.[55]

No significant effects of treatment with antimicrobial or antimicrobial-corticosteroid preparations on the growth quantity score of fungal colonies or fungal pathogens were found. However, significant effects were found of time on fungal population, indicating that perhaps changes in the local environment precipitated these effects. Prevalence of fungal habitation of the eye was significantly different within treatment groups at various time points. However, because all treated groups and the control eyes changed similarly, the differences could not be attributed to treatment effects. For instance, although fungal population varied somewhat prior to initiation of treatment, by week 2, fungal growth quantity scores were low in all 5 groups for both corneal and conjunctival specimens and remained low in all groups except corneas treated with NPD at week 4. The finding of comparable changes in each group, regardless of treatment, suggests that environmental factors and not effects of treatment were causative. Prevalence of ocular fungi isolated from a normal eye has been correlated with the immediate environment in a previous study[2] of ocular fungal population in hospitalized versus stabled equine patients. That study found a 2.5 times greater prevalence of fungi in stabled horses, compared with hospitalized horses. In our study, the dramatic changes in fungal populations in all 5 groups also illustrate the transient nature of fungal population of the equine eye, as the housing environment did not change during the study.

An association between presence of normal gram-positive flora and protection from overpopulation by potentially pathogenic bacteria and fungi[13,49] is supported by findings of our study. Namely, the growth quantity score of pathogens was low throughout the study period in all groups, but in treated eyes, it was observed that as positive culture results for gram-positive bacteria increased at week 2, positive culture results for gram-negative bacteria and fungi decreased. A *Streptomyces* sp, a common gram-positive isolate in our study, produces the efficacious antifungal agent natamycin.[2,6,43] However, *Streptomyces* spp were most commonly isolated prior to treatment when frequency of fungal isolation was greatest, and *Streptomyces* spp and fungi were both isolated at lower frequency at weeks 1, 2, and 4. Therefore, gram-positive effects on fungal flora in our study are more likely attributed to occupation of available space and use of nutrients than production of natural antifungal substances.

Corticosteroids added to the preparations did not appear to have adverse effects on the ocular flora. No increase in fungi or gram-negative bacteria, or, more specifically, bacterial or fungal species known to be pathogenic such as β-hemolytic *Streptococcus* spp, *P aeruginosa*, or *Aspergillus*, was found in eyes treated with preparations containing a corticosteroid (ie, Pred-G and NPD). Therefore, the role of topical corticosteroids in potentiation of infectious keratitis does not appear to be related to direct propagation of potential and known pathogens. Topical application of corticosteroids, antimicrobials, and their combinations has been shown clinically to potentiate or exacerbate fungal keratitis in humans, rabbits, and horses.[4,6,13,35-37,40,49] However, the effects of corticosteroids and antimicrobial-corticosteroid preparations are debatable in experimentally inoculated fungal contamination without corneal ulceration, in which some researchers have found an increase in fungal organisms with topical corticosteroid treatment,[35,63] whereas others have found no effect of treatment with topical antimicrobials or corticosteroids.[14] Corticosteroids potentiate corneal infection primarily by delay of healing and suppression of inflammation and phagocytic abilities of inflammatory cells.[41,44,46,47] These effects were not influential in our study because the eyes of this equine population were not injured or inflamed. It follows that a different result is seen clinically and experimentally in injured, inflamed, or infected eyes, in which corticosteroids inhibit epithelialization, vascularization, fibrosis, infiltration, and function of inflammatory cells and potentiate collagen breakdown.[41,44-47] Additionally, changes in the local corneal environment secondary to injury and infection (ie, decreased pH, oxygen content, and glucose concentration and increased lactic acid concentration and tissue damage by release of microbial and host extracellular products) may in conjunction with corticosteroid effects support an environment conducive to microbial propagation.[23,64,65] A final contributing factor to the deleterious effects of corticosteroids in corneal infection is the adjunctive use of antimicrobials with the corticosteroids, in which susceptible organisms composing the normal protective flora are eliminated, leaving potential and known pathogens and organisms with increased potential for resistance to commonly used antimicrobials. In the face of a corneal injury, treatment with a combination preparation can promote corneal degradation, delay healing, impair host defenses, and reduce the protective ocular flora. The result may be a devastating corneal infection by the remaining resistant potential pathogens. In light of these facts, topical antimicrobial-corticosteroid preparations should be used judiciously.

a. Bacitracin Zinc-neomycin-polymyxin B ointment, Fougera & Co, Melville, NY.
b. Pred-G, Allergan Pharmaceuticals, Hormigueros, Puerto Rico.
c. Bacitracin-neomycin-0.05% dexamethasone, Fougera & Co, Melville, NY.
d. LubriTears, Bausch & Lomb Pharmaceuticals Inc, Tampa, Fla.
e. Culture Swab Collection & Transport System, Diffco Laboratories, Detroit, Mich.
f. PROC-GLM in SAS, SAS Institute Inc, Cary, NC.
g. Sigma Stat, SPSS Science Inc, Chicago, Ill.

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