In vitro fungistatic and fungicidal activities of silver sulfadiazine and natamycin on pathogenic fungi isolated from horses with keratomycosis

Caroline M. Betbeze, DVM, MS; Ching Ching Wu, DVM, PhD; Sheryl G. Krohne, DVM, MS; Jean Stiles, DVM, MS

Objective —To evaluate the in vitro antifungal properties of silver sulfadiazine (SSD) and natamycin against filamentous fungi isolated from eyes of horses with keratomycosis.

Sample Population —Filamentous fungal isolates obtained from eyes of keratomycosis-affected horses.

Procedures —Fungal culture of ocular samples yielded 6 Fusarium spp; 7 Aspergillus spp; and 1 isolate each of Curvularia, Scopulariopsis, Penicillium, and Chrysosporium. For each fungal isolate, minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of SSD and natamycin were determined.

Results —For all 17 fungal isolates, SSD MIC distribution ranged from ≤1 to >64 μg/mL; MIC50 and MIC90 (MICs at which 50% and 90% of organisms were inhibited) were 4 and 32 μg/mL, respectively. The SSD MFC distribution for all isolates was ≤1 to >64 μg/mL; MFC50 and MFC90 (MFCs at which 50% and 90% of organisms were killed) were 8 and >64 μg/mL, respectively. For all fungal isolates, natamycin MIC distribution ranged from 256 to >1,000 μg/mL; MIC50 and MIC90 were 512 and >1,000 μg/mL, respectively. The natamycin MFC distribution for all isolates ranged from 512 to >1,000 μg/mL; MFC50 and MFC90 were each >1,000 μg/mL.

Conclusions and Clinical Relevance —These in vitro data suggest that SSD is fungicidal against the fungal isolates that were obtained from eyes of horses with keratomycosis and that natamycin is fungicidal against some of the isolates at the drug concentrations evaluated. Silver sulfadiazine may be a therapeutic option for equine keratomycosis.

Fungi are a normal component of the conjunctival microflora of horses but can cause disease following corneal injury. The most commonly isolated fungi from clinically normal equine conjunctiva are Aspergillus spp, Penicillium spp, Alternaria spp, Fusarium spp, and Cladosporium spp. The most commonly isolated fungi from the cornea of horses with keratomycosis are Aspergillus spp, Penicillium spp, Cladosporium spp, and Alternaria spp, although there are numerous cases reported in the veterinary medical literature in which less common filamentous fungi, such as species of Curvularia, Scopulariopsis, and Chrysosporium, were isolated.

For medical treatment of horses with keratomycosis, there are few effective options and 5% natamycin suspension is the only commercially available topical ophthalmic antifungal medication. Natamycin is a polyene antifungal antibiotic produced by Streptomyces natalensis. It possesses in vitro activity against species of Candida, Aspergillus, Cephalosporium, Fusarium, and Penicillium. Natamycin is predominantly fungicidal, but the activity against fungi is dose related. The mechanism of action appears to be mediated through binding of the drug molecule to the fungal cell membrane; the polyene-sterol complex alters membrane permeability, thereby depleting essential cellular constituents. In humans, topical use of natamycin is well tolerated, although a low-grade inflammation may develop with prolonged use.

Other commonly used medications for treatment of keratomycosis in humans and other animals include preparations of 1% miconazole, 0.2% fluconazole, and 1% itraconazole with 30% dimethyl sulfoxide and 1% silver sulfadiazine cream. Although the antibacterial properties of silver sulfadiazine have been extensively studied in vivo and in vitro, there is little informa-
tion regarding its antifungal properties. The antimicrobial activity is thought to be attributable to the reaction of silver with bacterial DNA and prevention of the unzipping of the double helix, which is the premise to microbial propagation. In humans, silver sulfadiazine cream has been used topically as an antifungal agent on infected skin wounds and as a treatment for keratomycosis. Some species of Candida (yeast) and Aspergillus are known to be inhibited by silver sulfadiazine in vitro, but fungicidal activity of the drug on these organisms and the methods of determination of fungistatic activity were not reported. Following topical application to eyes of rabbits (with and without corneal debridement), silver sulfadiazine (1%) cream is absorbed into the cornea, aqueous humor, and blood. These findings have led to the use of silver sulfadiazine in clinical cases of equine keratomycosis, although to our knowledge, silver sulfadiazine has not been evaluated for fungistatic or fungicidal activity against filamentous fungal pathogens isolated from keratomycosis-affected horses.

The purpose of the study reported here was to evaluate (by use of standardized methods) the in vitro antifungal properties of silver sulfadiazine and natamycin against filamentous fungi isolated from eyes of horses with keratomycosis. The antifungal activity of natamycin was selected for comparison with that of silver sulfadiazine because the former is the only currently commercially available ophthalmic antifungal medication.

Materials and Methods

Fungal organisms—Seventeen isolates of filamentous fungi previously obtained from horses with keratomycosis were evaluated in this study. Fungal samples were obtained from horses with keratomycosis in Indiana, Missouri, Georgia, Florida, and Alabama. These samples were collected by a variety of methods, including keratectomies, corneal scrapings, and corneal cultures. The numbers of horses and eyes from which these samples were obtained are not known. Specimens were cultured on SDA, and isolates were saved as pure strains on agar slants. The fungal isolates included 6 isolates of Fusarium spp; 7 isolates of Aspergillus spp; and 1 isolate each of Curvularia spp, Cladosporium spp, Scopulariopsis spp, Penicillium spp, and Chrysosporium spp. Speciation was not performed on these fungal isolates.

Susceptibility testing of fungal isolates—The protocol for broth dilution antifungal susceptibility testing of filamentous fungi described by the CLSI (formerly the National Committee for Clinical Laboratory Standards) was used for inoculum preparation and assessment of broth macrodilution susceptibility of filamentous fungi to antifungal agents.

To induce conidium and sporangiopore formation, all of the fungi evaluated were grown on SDA for 7 days at room temperature (approx 25°C). Suspensions of Aspergillus, Curvularia, Penicillium, Cladosporium, Scopulariopsis, and Chrysosporium isolates were prepared by adding 1 drop of Tween-20 and then 1 mL of sterile saline (0.88% NaCl) solution to the colonies. Fusarium isolates were prepared in a similar manner without the use of Tween-20. The colonies were gently probed with a transfer pipette. The resulting mixture was withdrawn and transferred to a sterile tube to suspend the conidia and hyphae in saline solution. Heavy particles were allowed to settle for 3 to 5 minutes. The upper homogeneous suspension was transferred to a sterile tube and vortexed for 15 seconds. Densities of the suspensions of Aspergillus, Curvularia, Penicillium, and Scopulariopsis isolates were determined by use of a spectrophotometer at a wavelength of 600 nm and adjusted with sterile saline solution to an optical density of 0.09 to 0.11 (80% to 82% transmittance). Similarly, suspensions of Fusarium spp were adjusted to an optical density of 0.15 to 0.17 (68% to 70% transmittance), and suspensions of Chrysosporium spp were adjusted to an optical density of 0.09 to 0.11 (80% to 82% transmittance). These suspensions were diluted 1:50 in RPMI-1640 with L-glutamine and without sodium bicarbonate and buffered at pH 7.0 with MOPS (0.165 mol/L). Each 1:50 inoculum suspension corresponded to 0.4 × 10^6 CFUs/mL to 5 × 10^6 CFUs/mL.

Reference grade powders of silver sulfadiazine and natamycin were used to prepare the drug stock solutions. A stock smooth emulsion of silver sulfadiazine (2 × 10^5 μg/mL) was prepared by stirring a weighed amount of powder with the appropriate volume of 60% (wt/wt) aqueous 1,2-propanediol (propylene glycol). This emulsion was then sonicated for 1 minute at room temperature. The stock solution was stored at room temperature and protected from exposure to light. Subsequent dilutions of the emulsion were made in growth medium (RPMI-1640 buffered with MOPS) at the time of testing and were used immediately. A stock solution of natamycin (2,000 μg/mL) was prepared by dissolving of a weighed amount of reference-grade powder in 0.5N sodium hydroxide. Within 30 minutes, the solution was neutralized with 0.5N hydrochloric acid. The stock solution was stored at 4°C and protected from exposure to light; dilutions were made in RPMI-1640 medium buffered with MOPS immediately before use.

Ten dilutions of the silver sulfadiazine stock solution were made in RPMI-1640 growth medium; final concentrations were 25,000, 12,500, 6,250, 64, 32, 16, 8, 4, 2, and 1 μg/mL. The higher dilutions for silver sulfadiazine that were used were based on the concentration of the commercially available cream (1%). The lower dilutions of silver sulfadiazine were based on published reports of the MIC ranges of silver sulfadiazine against bacteria because the MIC ranges of silver sulfadiazine against fungi are currently unknown. Seven serial dilutions of natamycin stock solution were made in the RPMI-1640 growth medium; final concentrations were 1,000, 512, 256, 128, 64, 32, and 16 μg/mL. The dilutions for natamycin were chosen on the basis of published MIC ranges for natamycin against yeasts and filamentous fungi. Aliquots of 0.5 mL of each drug dilution were placed in 8-mL sterile, polystyrene, round-bottomed tubes and vortexed with 0.5 mL of fungal suspension for 3 to 5 seconds. A drug-free growth tube and a tube containing only growth medium were included for each fungal isolate as positive and negative control specimens, respectively. In addition, the solvent used for the silver sulfadiazine suspension (propylene glycol) was also diluted with growth medium and then mixed with the fungal suspensions to determine whether this solvent could cause fungal growth inhibition without the addition of the antifungal drug. To minimize error, the macrodilution procedure was carried out in duplicate for each fungal isolate. The tubes were incubated at 36 ± 1°C for 48 to 72 hours without agitation. Tubes were visually inspected for growth and compared with the positive control tube. A 5-point turbidity scale was used to assess fungal growth (compared with that of the positive control tube) as follows: 4, no reduction in growth; 3, slight reduction in growth or growth equivalent to approx 75% of the growth in the positive control tube (drug-free medium); 2, prominent growth; 1, identical growth to that of the positive control tube; 0, no growth. Each 1:50 inoculum suspension corresponded to 0.4 × 10^6 CFUs/mL to 5 × 10^6 CFUs/mL.
reduction in growth or growth equivalent to approximately 50% of the growth in the positive control tube; 1, slight growth or growth equivalent to approximately 25% of the growth in the positive control tube; and 0, optically clear or absence of growth. The end points for polyene antifungal agents (natamycin) are typically not trailing, and the MIC is determined as the lowest drug concentration that prevents any discernible growth (score 0). To our knowledge, a break point has not been determined for silver sulfadiazine because it has not been evaluated with this standardized method. For this study, the break point was set as the lowest drug concentration that prevented any discernible growth (score 0) for both natamycin and silver sulfadiazine. Because silver sulfadiazine is a suspension and consequently has a cloudy visual appearance in dilutions > 64 μg/mL, all silver sulfadiazine drug dilutions were plated on SDA after being visually inspected for growth to determine whether growth was truly inhibited. The MIC<sub><0.50></sub> and MIC<sub><90></sub> values were determined for all fungal isolates as a group.

In vitro fungicidal activities were determined for silver sulfadiazine and natamycin by use of a previously published method. Twenty-microliter aliquots from each tube in which growth was completely inhibited (i.e., tubes that were visually clear), from the tube with the lowest drug dilution at which growth occurred, and from the negative control tube (drug-free medium) were subcultured onto SDA plates. Because silver sulfadiazine is a cloudy suspension at concentrations > 64 μg/mL, all silver sulfadiazine drug dilutions were plated on SDA after being visually inspected for growth to determine whether growth was truly inhibited. The MIC<sub><50></sub> and MIC<sub><90></sub> values were determined for all fungal isolates as a group.

### Results

At 48 or 72 hours, depending on the growth rate of the positive control specimen, the tubes were visually inspected for growth and the MIC values were determined for each fungal isolate. For silver sulfadiazine, the MIC distribution for all 17 fungal isolates ranged from ≤ 1 to > 64 μg/mL. For Fusarium spp (n = 6), the MIC distribution ranged from ≤ 1 to > 64 μg/mL. For Aspergillus spp (n = 7), the MIC distribution ranged from 4 to 8 μg/mL, and the majority (6) of the isolates was inhibited at a concentration of 4 μg/mL. For the group of other fungal isolates (n = 4), the MIC distribution ranged from ≤ 1 to > 4 μg/mL. (Table 1). Among all fungal isolates, the MIC<sub><50></sub> and MIC<sub><90></sub> for silver sulfadiazine were 4 and 32 μg/mL, respectively. For silver sulfadiazine, the MIC distribution for all 17 fungal isolates ranged from ≤ 1 to > 64 μg/mL. For Aspergillus spp (n = 7), the MIC distribution ranged from 4 to > 64 μg/mL. For Fusarium spp (n = 6), the MIC distribution ranged from 2 to > 64 μg/mL. For Aspergillus spp (n = 7), the MIC distribution ranged from 4 to > 64 μg/mL. For the group of other fungal isolates (n = 4), the MIC distribution ranged from ≤ 1 to > 64 μg/mL. (Table 2). Among all fungal isolates, the MIC<sub><50></sub> and MIC<sub><90></sub> for silver sulfadiazine were 8 and > 64 μg/mL, respectively. When 1:8, 1:16, 1:32, and 1:64 dilutions of the solvent for silver sulfadiazine (propylene glycol) were mixed with fungal suspensions, fungal growth was visually similar to that of the positive control tubes for each fungal isolate. These propylene glycol-fungal suspensions were also plated on SDA; the number of colonies that grew from those samples was similar to the number that grew in the positive control tubes.

For natamycin, the MIC distribution for all 17 fungal isolates ranged from 256 to > 1,000 μg/mL. For Fusarium spp (n = 6), the MIC distribution ranged from 512 to 1,000 μg/mL. For Aspergillus spp (n = 7), the MIC distribution ranged from 512 to 1,000 μg/mL. For the group of other fungal isolates (n = 4), the MIC distribution ranged from 256 to 1,000 μg/mL. (Table 3). Among all fungal isolates, the MIC<sub><50></sub> and MIC<sub><90></sub> were 512 and > 1,000 μg/mL, respectively. The MIC distribution for all 17 fungal isolates ranged from 512 to 1,000 μg/mL. For Fusarium spp (n = 6), the MIC distribution ranged from 512 to > 1,000 μg/mL. For Aspergillus spp (n = 7), the MIC distribution ranged from 512 to > 1,000 μg/mL. For the group of other fungal isolates (n = 4), the MIC distribution ranged from 256 to > 1,000 μg/mL. (Table 4). Among all fungal isolates, the MIC<sub><50></sub> and MIC<sub><90></sub> were 512 and > 1,000 μg/mL, respectively. The MIC distribution for all 17 fungal isolates ranged from 512 to > 1,000 μg/mL. For Fusarium spp (n = 6), the MIC distribution ranged from 512 to > 1,000 μg/mL. For Aspergillus spp (n = 7), the MIC distribution ranged from 512 to > 1,000 μg/mL. For the group of other fungal isolates (n = 4), the MIC distribution ranged from 256 to > 1,000 μg/mL.
Discussion

The study reported here was performed to determine whether the antifungal efficacy attributed to silver sulfadiazine in the treatment of keratomycosis in humans and horses (in anecdotal reports and reported findings of clinical trial) could be affirmed via traditional antifungal susceptibility testing. Results of the present study indicated that silver sulfadiazine inhibited and killed all of the fungal isolates (obtained from eyes of horses with keratomycosis) that were evaluated with a standardized CLSI testing method. Natamycin also had fungicidal activity against some of the fungi evaluated, but some of the Fusarium isolates were resistant to natamycin at the drug concentrations used.

Although the antifungal activity of silver sulfadiazine was not evaluated in vivo, the results of our study correlate well with the findings of clinical trials in which silver sulfadiazine was used to treat keratomycosis in humans. In vivo studies of antifungal medications are important because host factors, such as drug pharmacokinetic factors, host immune status, and site and severity of infection, seem to have as much value as the MIC as predictors of clinical outcome of treatment.

was 512 μg/mL for the 2 fungal isolates that grew; 2 fungal isolates did not grow when plated (Table 4). Among all fungal isolates, the MFC50 and MFC90 values for natamycin were each >1,000 μg/mL.

For silver sulfadiazine and natamycin, the MFC and MIC ranges of each drug were similar for each group of fungi. For silver sulfadiazine, the MFC50 value differed from the MIC50 by only 1 drug dilution, as did the MFC90 value, compared with the MIC90 value (Tables 1 and 2). For natamycin, the MFC50 and MFC90 values could not be compared to the MIC50 and MIC90 values because the MFC90 and MIC90 values were both >1,000 μg/mL; therefore, the value at which natamycin would be fungicidal could not be determined at the drug concentrations that were evaluated.

Table 3—Minimum inhibitory concentration distribution* of natamycin against various fungal isolates obtained from eyes of horses† with keratomycosis.

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>MIC value (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt; 1,000</td>
</tr>
<tr>
<td>Fusarium spp (n = 6)</td>
<td>4</td>
</tr>
<tr>
<td>Aspergillus spp (7)</td>
<td>0</td>
</tr>
<tr>
<td>Other§ (4)</td>
<td>0</td>
</tr>
<tr>
<td>All fungal isolates</td>
<td>4</td>
</tr>
</tbody>
</table>

See Table 1 for key.

Table 4—Minimum fungicidal concentration distribution* of natamycin against various fungal isolates obtained from eyes of horses† with keratomycosis.

<table>
<thead>
<tr>
<th>Fungal isolate</th>
<th>MFC value (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&gt; 1,000</td>
</tr>
<tr>
<td>Fusarium spp (n = 8)</td>
<td>5</td>
</tr>
<tr>
<td>Aspergillus spp (7)</td>
<td>3</td>
</tr>
<tr>
<td>Other§ (2)</td>
<td></td>
</tr>
<tr>
<td>All fungal isolates</td>
<td>8</td>
</tr>
</tbody>
</table>

(O) the 4 isolates in this category, 2 failed to grow. See Tables 1 and 2 for remainder of key.
fungal growth inhibition have not been published for silver sulfadiazine, our determination that silver sulfadiazine was fungistatic was based on a break point that was set as the lowest drug concentration that prevented any discernible growth of fungi. Natamycin, however, has been evaluated in previous research and was found to be fungistatic against 17 of 22 fungal isolates obtained from horses with keratomycosis, although a different method (assessment of IC₅₀ [amount of drug that reduces growth of the tested fungus to 50% or less of the amount of growth in the drug-free control culture]) was used to make those determinations. In the present study, the MIC₅₀ and MIC₉₀ for natamycin were 512 and >1,000 \( \mu \text{g/mL} \), respectively. Four of the 6 Fusarium isolates were resistant to natamycin at a concentration of 1,000 \( \mu \text{g/mL} \), which was the highest concentration evaluated. Fungal isolates that were evaluated in our study may have been more resistant to natamycin than isolates in the study by Brooks et al because the break point determinations in the CSLI method are based on 100% inhibition of growth.

Many studies have revealed that differences in inoculum preparation and media, as well as incubation time and temperature, can influence results of antifungal susceptibility testing. For this reason, a CLSI subcommittee has standardized MIC data derived from in vitro testing by standardizing inoculum size and preparation, incubation time and temperature, media, and end-point determination. The CLSI method was used in the present study because it is a standardized testing method and would allow results to be easily compared with findings of other studies from different laboratories. Because we did not know which concentration of silver sulfadiazine would be inhibitory, a wide range of drug concentrations was evaluated to determine the MIC of silver sulfadiazine, including the concentration that is commercially available (1%). Our data indicated that silver sulfadiazine was effective in vitro against all of the evaluated fungal isolates at a much lower concentration range than the concentration provided in the commercially available preparation. It was also a concern that the solvent for silver sulfadiazine (propylene glycol) may inhibit fungal growth. Because of this concern, the concentrations of propylene glycol that were used to suspend silver sulfadiazine in the present study were assessed for fungal inhibition without drug but in the same manner as when mixed with silver sulfadiazine.

In the cornea, yeasts and filamentous fungi are present only in their filamentous phases; therefore, it was important that fungi in their filamentous forms were evaluated in the present study. The antifungal activity of silver sulfadiazine has been evaluated in vitro, but the methods were not standardized or the fungal isolates were not filamentous. In a previous investigation, it was determined that silver sulfadiazine is absorbed by the rabbit cornea through either debrided or intact corneal epithelium, although the silver sulfadiazine corneal concentration is approximately 10 times as high when the corneal epithelium has been debrided. The fact that silver sulfadiazine is absorbed into the cornea and aqueous humor, has been used successfully in treatment of fungal keratitis in humans, and is fungistatic and fungicidal against filamentous fungi in vitro (as indicated by the findings of the present study) suggest that silver sulfadiazine may be useful in treatment of equine keratomycosis.

Natamycin penetrates intact corneal epithelium poorly, but is well absorbed through an ulcerated cornea when administered frequently. Although natamycin has been thought to have poor corneal penetration and low bioavailability in the cornea, it has been extensively used in the treatment of keratomycosis in humans and horses and has been effective. Resistance to polyenes such as natamycin is unusual but not unknown. Ziogas et al analyzed the sterol content in natamycin-resistant Aspergillus nidulans mutants and found that these isolates had a low ergosterol content that could possibly result in resistance to natamycin. There may also be other mechanisms that result in resistance to polyenes because some mutants in that study had a normal ergosterol content and were nevertheless resistant. It is possible that the Fusarium isolates that were resistant to natamycin in the present study could have been deficient in ergosterol, thereby causing natamycin to be ineffective. It is interesting to note that the same isolates were susceptible to silver sulfadiazine, which is thought to kill microorganisms by unzipping the DNA helix. It may also be possible that these isolates could have been susceptible to natamycin at a drug concentration higher than those evaluated in our study.

Minimum fungicidal concentration for silver sulfadiazine and natamycin was also investigated. Again, because MFC has not been evaluated for silver sulfadiazine or natamycin, there were no previously determined values with which to compare the results of our study. It is important to note that the MFC ranges of silver sulfadiazine differed from the MIC ranges by approximately 1 drug dilution for the group of Fusarium spp and by approximately 2 drug dilutions for Aspergillus spp. This close association between MIC and MFC has been reported to indicate primary fungicidal activity when other antifungal drugs have been studied in this manner. Minimum fungicidal concentration could not be determined for natamycin for some of the Fusarium isolates because they were not inhibited by any of the concentrations of natamycin that were evaluated. The isolates on which the MFC determination was performed were within 1 to 2 drug dilutions of the MICs for these isolates, which indicates primary fungicidal activity.

With consideration of the scope of the present study, these data support the use of silver sulfadiazine in keratomycosis-affect ed horses. Natamycin may be an effective treatment for horses with keratomycosis, but some isolates of Fusarium spp were resistant to this drug in vitro. This finding should be taken into consideration when choosing a treatment as the first line of defense against keratomycosis. In vitro antifungal susceptibility testing is valuable in determination of which fungi are resistant to the drugs under evaluation, but it is still not possible to predict a consistently reliable clinical outcome for silver sulfadiazine or natamycin on the basis of in vitro results. To support our in vitro findings, in vivo studies of the antifungal
activity of silver sulfadiazine in horses with keratomycosis are warranted.


b. University of Georgia, University of Florida, Auburn University, University of Missouri-Columbia, and Purdue University.

c. Spectronic 20+, Spectronic Instruments, Rochester, NY.

d. GIBCO-BRL Life Technologies, Easa, Spain.

e. Spectrum Chemicals and Laboratory Products, Gardena, Calif.

f. US Pharmacopea, Rockville, Md.

References


