The combined utilization of Chlorhexidine and Voriconazole or Natamycin to combat Fusarium infections

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Abstract

Background: Fusarium species are the fungal pathogens most commonly responsible for the mycotic keratitis, which are resistant to the majority of currently available antifungal agents. The present study was designed to assess the efficacy of a combination of low doses chlorhexidine with two other commonly used drugs (voriconazole and natamycin) to treat Fusarium infections.

Results: We utilized combinations of chlorhexidine and natamycin or voriconazole against 20 clinical Fusarium strains in vitro using a checkerboard-based microdilution strategy. In order to more fully understand the synergistic interactions between voriconazole and chlorhexidine, we utilized a Galleria mellonella model to confirm the combined antifungal efficacy of chlorhexidine and voriconazole in vivo. We found that for voriconazole, natamycin, and chlorhexidine as single agents, the minimum inhibitory concentration (MIC) ranges were 2–8, 4–16, and >16 μg/ml, respectively. In contrast, the MIC values for voriconazole and chlorhexidine were reduced to 0.25–1 and 1–2 μg/ml, respectively, when these agents were administered in combination, with synergy being observed for 90% of tested Fusarium strains. Combined chlorhexidine and natamycin treatment, in contrast, exhibited synergistic activity for only 10% of tested Fusarium strains. We observed no evidence of antagonism. Our in vivo model results further confirmed the synergistic antifungal activity of chlorhexidine and voriconazole.

Conclusions: Our results offer novel evidence that voriconazole and chlorhexidine exhibit synergistic activity when used to suppress the growth of Fusarium spp., and these agents may thus offer value as a combination topical antifungal treatment strategy.

Keywords: Fusarium, Chlorhexidine, Voriconazole, Natamycin, Synergistic

Background

Keratomycosis is a form of fungal infection that can be challenging to treat, and that can result in permanent and severe vision damage when not adequately treated [1]. Fusarium species are the causative pathogens in between 36 and 67% of all traumatic keratitis cases, in which F. oxysporum was the most frequently isolated species followed by F. solani [2]. Fusarium-related keratitis remains challenging to treat as these fungi are intrinsically resistant to most available antifungal agents. While advances in the standard treatment for keratitis have been developed in recent years, with natamycin (NAT) and voriconazole (VOR) being the current treatment agents of choice, further optimization of these therapeutic regimens is still warranted [3].

While previous studies have shown that NAT can be effective for the treatment of Fusarium infections, and 5% NAT is currently the first-line treatment for mycotic
keratitis in certain nations, the poor penetration of this compound has been linked to failed treatment in some cases [1]. More recently, the application of 1% topical VOR has been shown to be an effective means of treating refractory fungal keratitis [4] while also exhibiting satisfactory diffusion within the aqueous humor. However, single-agent VOR treatment has not been shown to be adequately protective as a means of treating some patients, suggesting that combination therapies may be necessary to achieve reliable and durable therapeutic efficacy [5]. Chlorhexidine (CHL) is an antiseptic agent that is commonly used and has been shown to be safe for ophthalmic exposure at concentrations of 1% or below [6]. Furthermore, the intravitreal injection of 0.1% CHL has been shown to be a safe antiseptic strategy [7]. The utility of CHL for treating keratomycosis, however, remains to be established.

The goal of the present study was to assess the impact of combinations of NAT or VOR with CHL on clinical Fusarium isolates. We employed a checkerboard microdilution strategy to reliably identify potentially useful combinations of these therapeutic agents in vitro. We then employed a G. mellonella model to validate our findings in vivo.

### Results

#### Assessment of the in vitro antifungal activity of CHL, NAT, and VOR

CHL, VOR, and NAT solutions exhibited Minimum inhibitory concentration (MIC) values of >16 μg/ml, 2–8 μg/ml, and 4–16 μg/ml to the Fusarium isolates, respectively. CHL did not exhibit antifungal activity, even at the highest tested concentration. When combined with VOR, the MIC values for CHL and VOR were reduced to 1–2 μg/ml and 0.25–1 μg/ml respectively, with synergy being observed for 18 Fusarium strains (90%). In contrast, such synergistic interactions were only observed for 2 Fusarium strains (10%) treated with a combination of CHL and NAT (Table 1). We did not observe any evidence of antagonism in any of these analyses.

#### Assessment of the in vivo antifungal activity of CHL and VOR in G. mellonella

In order to evaluate the synergistic efficacy of CHL and VOR in vivo, we infected G. mellonella with F. solani Jfs1 and then treated these larvae using CHL and/or VOR. The survival in groups treated with VOR, CHL,

### Table 1 Combination activity of CHL with VOR or NAT against Fusarium species

| Strains | Origin          | MICs (μg/ml) | FICI | MICs (μg/ml) |
|---------|-----------------|-------------|------|-------------|
|         | CHL | VOR | CHL/VOR | FICI | NAT | CHL/NAT | FICI |
| F. Solani |     |     |        |      |     |         |      |
| Jfs1    | Skin         | > 16        | 4     | 1/0.5 | SYN | 8     | 2/8  | N     |
| Jfs2    | Skin         | > 16        | 2     | 1/0.5 | SYN | 8     | 2/8  | N     |
| Jfs3    | Cornea       | > 16        | 2     | 1/1   | N    | 8     | 1/8  | N     |
| Jfs4    | Cornea       | > 16        | 8     | 2/0.5 | SYN | 4     | 2/4  | N     |
| Jfs5    | Skin         | > 16        | 2     | 2/0.5 | SYN | 8     | 2/8  | N     |
| Jfs6    | Cornea       | > 16        | 4     | 1/0.5 | SYN | 16    | 2/4  | SYN   |
| Jfs7    | Skin         | > 16        | 4     | 1/0.5 | SYN | 8     | 1/4  | N     |
| Jfs8    | Auditory canal | > 16   | 2     | 1/0.5 | SYN | 8     | 1/4  | N     |
| Jfs9    | Auditory canal | > 16  | 2     | 1/0.5 | SYN | 8     | 1/4  | N     |
| Jfs10   | Nail         | > 16        | 2     | 2/0.5 | SYN | 4     | 2/4  | N     |
| Jfs11   | Nail         | > 16        | 2     | 1/0.5 | SYN | 8     | 1/4  | N     |
| Jfs12   | Cornea       | > 16        | 4     | 1/0.5 | SYN | 8     | 1/4  | N     |
| Jfo1    | Cornea       | > 16        | 2     | 1/0.25 | SYN | 8     | 2/4  | N     |
| Jfo2    | Nail         | > 16        | 4     | 2/0.5 | SYN | 8     | 1/8  | N     |
| Jfo3    | Skin         | > 16        | 4     | 2/0.5 | SYN | 8     | 1/2  | SYN   |
| Jfo4    | Skin         | > 16        | 2     | 1/1   | N    | 8     | 2/8  | N     |
| Jfo5    | Cornea       | > 16        | 2     | 1/0.25 | SYN | 8     | 2/4  | N     |
| Jfo6    | Nail         | > 16        | 2     | 1/0.5 | SYN | 4     | 1/4  | N     |
| Jfo7    | Auditory canal | > 16  | 2     | 1/0.5 | SYN | 8     | 1/4  | N     |
| Jfo8    | Nail         | > 16        | 2     | 1/0.5 | SYN | 8     | 1/4  | N     |

SYN synergy (FICI ≤0.5), N indifference (no interaction 0.5 < FICI≤4), CHL Chlorhexidine, VOR Voriconazole, NAT Natamycin, MIC minimum inhibitory concentration, FICI Fractional inhibitory concentration index

### Table 2 Competition activity of CHL with VOR or NAT against Fusarium species

| Strains | Origin          | MICs (μg/ml) | FICI | MICs (μg/ml) |
|---------|-----------------|-------------|------|-------------|
|         | CHL | VOR | CHL/VOR | FICI | NAT | CHL/NAT | FICI |
| F. oxysporum |     |     |        |      |     |         |      |
| Jfo1    | Cornea       | > 16        | 2     | 1/0.25 | SYN | 8     | 2/4  | N     |
| Jfo2    | Nail         | > 16        | 4     | 2/0.5 | SYN | 8     | 1/8  | N     |
| Jfo3    | Skin         | > 16        | 4     | 2/0.5 | SYN | 8     | 1/2  | SYN   |
| Jfo4    | Skin         | > 16        | 2     | 1/1   | N    | 8     | 2/8  | N     |
| Jfo5    | Cornea       | > 16        | 2     | 1/0.25 | SYN | 8     | 2/4  | N     |
| Jfo6    | Nail         | > 16        | 2     | 1/0.5 | SYN | 4     | 1/4  | N     |
| Jfo7    | Auditory canal | > 16  | 2     | 1/0.5 | SYN | 8     | 1/4  | N     |
| Jfo8    | Nail         | > 16        | 2     | 1/0.5 | SYN | 8     | 1/4  | N     |

SYN synergy (FICI ≤0.5), N indifference (no interaction 0.5 < FICI≤4), CHL Chlorhexidine, VOR Voriconazole, NAT Natamycin, MIC minimum inhibitory concentration, FICI Fractional inhibitory concentration index
and VOR with CHL was 15%, 10% and 33.3%, respectively. VOR treatment slightly improved larval survival, whereas CHL alone failed to improve larval survival, compared with the conidia group. Treatments with VOR combined with CHL significantly (P < 0.05) prolonged the survival of larvae (Fig. 1). Together, these in vivo findings thus confirmed the synergistic antifungal activity of CHL and VOR as evidenced by improved larval survival.

Histopathological analyses
On day 3 post- \textit{F. solani} Jzfs1 infection, we conducted a histopathological assessment of larvae in this study. We observed the formation of \textit{F. solani} spore and hyphae clusters in infected tissues regardless of treatment status (Fig. 2). There were 6, 4, 3 and 2 visible fungal clusters in the control group (Fig. 2a and e), CHL group (Fig. 2b and f), VOR group (Fig. 2c and g) and CHL + VOR group (Fig. 2d and h), respectively. VOR treatment was associated with a slight reduction of the number of visible fungal clusters relative to control and CHL groups. The combination treatment group exhibited dramatic reductions of the number of visible fungal clusters relative to the other three evaluated groups.

Discussion
Herein we evaluated the potential combination antifungal activity of antifungal agents against pathogenic \textit{Fusarium} spp. CHL, which is a common, inexpensive, safe, and efficacious antiseptic agent exhibited promising performance. CHL is functions by binding to cell membranes and thereby impairing bacterial adhesion and driving the leakage of bacterial cellular contents [8]. Most bacterial and \textit{Candida} species have been shown to be killed by 1–2% CHL solutions [9], while CHL concentrations of 1% or lower have been shown to be safe when used for ophthalmic purposes [10]. There are several studies showing that CHL exhibits in vitro antifungal activity against \textit{Fusarium} spp. Oliveira et al. found that CHL exhibited fungicidal activity against 90% of tested \textit{F. oxysporum} strains and 100% of tested \textit{F. solani} strains when evaluating 98 strains isolated from fungal keratitis patients [11]. Xu et al. found that the MIC range for CHL is 8–32 μg/mL, while the MIC₉₀ value of chlorhexidine was 32 μg/mL for 24 \textit{F. solani} strains [12]. In the Netherlands between 2005 and 2016, 89 cases of \textit{Fusarium} keratitis from 16 different hospitals were identified, and in vitro susceptibility testing indicated that chlorhexidine was active against \textit{Fusarium} spp. with a MIC range of 8–32 mg/L for \textit{F. solani} and 1–64 mg/L for \textit{F. oxysporum} [5]. In our study, we found that CHL did not cause any detectable inhibition of \textit{Fusarium} species when used as a single agent at the highest tested concentration (MIC > 16 μg/mL). The difference between our findings and these prior studies may be attributable to the fact that relatively few fungal isolates were tested and that lower concentrations of CHL were employed herein.

In two blinded randomized trials by the same investigators, they observed patients of fungal keratitis treated with natamycin compared to CHL gluconate at various concentrations. Their results indicated that 0.2% CHL yielded the best results [13, 14]. However, the overall estimate of effect was uncertain [15]. Fungal keratitis caused by \textit{F. solani} has been successfully treated with a combination of 0.02% CHL and AMB (Amphotericin B), underscoring the potential of CHL as an approach to the clinical management of fungal keratitis [16]. However, data regarding treatment with a combination of CHL and VOR is still limited.

In this study, we evaluated therapeutic interactions between CHL and VOR or NAT via a checkerboard microdilution strategy. As a first-line drug used for the management of fungal keratitis, NAT exhibited poor synergy ability with CHL both in vitro and in vivo. In
contrast, the combination of CHL and VOR treatment exhibited synergistic activity against 90% of tested *Fusarium* strains. We observed no evidence of antagonism. As *G. mellonella* exhibit immunological response similar to those of mammals and are easy to manipulate, can be maintained at low costs, and incur minimal ethical concerns [17], they can be used as an ideal model system for studies of fungal virulence and antifungal drug activity [18]. We therefore utilized a *G. mellonella* model to evaluate the in vivo synergistic activity of these treatments. We determined that combination VOR + CHL treatment of infected *G. mellonella* larvae was associated with significant increases in larval survival. What’s more, the concentration of CHL is about 0.00015%, which is very low and proved to be safe when used in ophthalmic [10]. The mechanisms underlying this synergy are likely attributable to the ability of CHL to increase VOR penetration and/or to direct damage to *Fusarium* cell membranes without affecting drug efflux pump activity [19].

In order to expand upon these in vivo studies, we additionally conducted microscopic analyses of infected larvae (Fig. 2), which confirmed that combination treatment was associated with a reduction in the degree of tissue damage observed in *G. mellonella* larvae relative to control groups, suggesting that these two compounds exhibit excellent synergy in vitro and in vivo and are thus ideal for treating *Fusarium* keratitis.

**Conclusions**

Our results demonstrate that CHL and VOR exhibit synergistic efficacy against *Fusarium* species in vitro and in vivo. These findings suggest that CHL and VOR may be a viable therapeutic combination treatment for *Fusarium* infections, although future clinical trials and studies will be needed to validate this finding and to explore the underlying molecular mechanisms.

**Methods**

**Fungal strains**

In total, we obtained 20 clinical *Fusarium* isolates (12 *F. solani* and 8 *F. oxysporum* strains) from clinical cultures (Table 1). These fungi were identified based upon a combination of morphological analyses and sequencing of the internal transcribed spacer (ITS) rDNA and translation elongation factor (TEF) 1α coding regions [20].

**Antifungal agents**

VOR (purity≥99%), NAT (purity≥99%) and CHL (purity≥99%) were obtained as powders from Selleck Chemicals (TX, USA), and were dissolved with DMSO (Amresco, OH, USA) to prepare 1600 μg/mL stock solutions.

**Inoculum preparation**

*Fusarium* strains were grown for 7 days at 30°C on Sabouraud dextrose agar (SDA), after which they were isolated and resuspended in a 2 mL volume of sterile saline. Sterile gauze was utilized to filter conidia suspensions, after which a hemocytometer was used to quantify concentrations therein, which were adjusted to 1–5 × 10⁶ cfu/mL.

**Assessment of single-agent antifungal activity in vitro**

We conducted antifungal susceptibility tests based upon the CLSI M38-A2 [21]. Briefly, stock solutions were diluted in a two-fold serial manner using RPMI-1640 (Gibco, NY, USA) to yield final concentrations of 0.0313–16 μg/mL. Microdilution wells were then filled with 100 μL of appropriate *Fusarium* isolates at 1–5 × 10⁴ cfu/mL. Plates were incubated for 48 h at 35°C, after which MIC values were determined by identifying the minimum antifungal agent dose necessary to achieve 100% inhibition of fungal growth relative to control
untreated wells. We additionally included *A. flavus* strain ATCC 204304 as a quality control strain in the present analysis.

**Assessment of in vitro interactions between CHL and VOR or NAT**

A checkerboard microdilution strategy was used to evaluate synergistic interactions between CHL and NAT or VOR against *Fusarium* strains, with this approach having been adapted from the CLSI M38-A2 microdilution method. Briefly, we added 50 μL volumes of serially-diluted VOR or NAT horizontally across microdilution plates, while 50 μL CHL samples that had been serially diluted were added in a vertical direction, with 100 μL of a prepared inoculum suspension also being added to each well. Incubation times and MIC determinations for this assay were as above. Combination drug interactions were classified based upon the fractional inhibitory concentration index (FICI) [22] which was calculated as follows: FICI = (MIC A in combination/MIC A alone) + (MIC B in combination/MIC B alone). Synergy was said to exist if FICI was ≤0.5, while the interaction was said to be indifferent when FICI was > 0.5 and < 4.0, and antagonistic when FICI was ≥4.0. Assays were conducted in duplicate on different days to ensure validity.

**G. mellonella survival assays**

*Galleria mellonella* caterpillars from the final instar larval developmental stage (Chengdu Pets and Insects Company, Sichuan, China) were maintained under dark conditions and were utilized within 1 week of receipt. In total, 20 randomly selected larvae (330 ± 25 mg, 2–3 cm) were utilized per group. Two control groups were injected with 10 μL of saline or with no solution, respectively. Infected animals were injected with a 10 μL volume containing *Fusarium Jzfs1* (1 × 10^7/mL) using a 25 μL Hamilton syringe. Injections were made into the hemocoel of each larva through the last left proleg, with hematoxylin and eosin (HE). Samples were then dehydrated with an ethanol gradient (70, 80, 90, 96, and 100% ethanol). Samples were then paraffin and xylene embedded, sliced to prepare 8 μm sections, and stained with hematoxylin and eosin (HE). Samples were then evaluated via an FSX100 fluorescence microscope (Olympus, Tokyo, Japan) at 10× and 40×. As controls, saline-injected larvae were also collected.

**Histological analyses**

*Fusarium* presence within *G. mellonella* tissues was assessed via collecting larvae from each group on day 3 post-infection and treatment. These larvae were fixed with 10% neutral formalin, after which they were dehydrated with an ethanol gradient (70, 80, 90, 96, and 100% ethanol). Samples were then paraffin and xylene embedded, sliced to prepare 8 μm sections, and stained with hematoxylin and eosin (HE). Samples were then evaluated via an FSX100 fluorescence microscope (Olympus, Tokyo, Japan) at 10× and 40×. As controls, saline-injected larvae were also collected.

**Abbreviations**

MIC: Minimum inhibitory concentration; FICI: Fractional inhibitory concentration index; NAT: Natamycin; VOR: Voriconazole; CHL: Chlorhexidine; AMB: Amphotericin B; ITS: Internal transcribed spacer; TEF: Translation elongation factor

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**Authors’ contributions**

TJ and JT carried out the in vitro and in vivo antifungal experiment, ZW and TJ collection and analysis the experiment data, JWT designed, interpreted the experiment data and wrote the manuscript, YS and LY revised the manuscript critically for important content. All authors read and approved the final manuscript.

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**Availability of data and materials**

All data generated or analyzed during this study are included in this published article. Access to raw data can be acquired by connecting to the corresponding author via email.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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References
1. Mahmoudi S, Masoomi A, Ahmadikia K, Tabatabaei SA, Soleimani M, Rezaie S, Ghazchehian H, Barafsheh-Afshani A. Fungal keratitis: an overview of clinical and laboratory aspects. Mycoses. 2018;61(12):916–30.
2. Fekih O, Haj Said O, Zgolli HM, Mabrouk S, Bakir K, Nacef L. Microbiologic profile of the mycosic abscess on a reference center in Tunisia. Tunis Med. 2019;97(5):644–9.
3. Austin A, Liemtan T, Rose-Nussbaumer J. Update on the Management of Infectious Keratitis. Ophthalmology. 2017;124(11):1676–89.
4. Sharma S, Das S, Virdi A, Fernandes M, Sahu SK, Kumar Koday N, Ali MH, Garg P, Motukupally SR. Re-appraisal of topical 1% voriconazole and 5% natamycin in the treatment of fungal keratitis in a randomised trial. Br J Ophthalmol. 2015;99(9):1190–5.
5. Oliveira Dos Santos C, Kolwijck E, van Rooij J, Stoumenbeek R, Visser N, Cheng YY, NTY S, Verweij PE, Eggink CA. Epidemiology and Clinical Management of Fusarium keratitis in the Netherlands, 2005–2016. Front Cell Infect Microbiol. 2020;10:133.
6. Oakley C, Allen P, Hooshmand J, BJT V. Pain and antisepsis after ocular administration of povidone-iodine versus chlorhexidine. Retina. 2018;38(10):2064–6.
7. Oakley CL, Vote BJ. Aqueous chlorhexidine (0.1%) is an effective alternative to povidone-iodine for intravitreal injection prophylaxis. Acta Ophthalmol. 2016;94(8):e808–9.
8. Letzelter J, Hill JB, Hacquebord J. An overview of skin antiseptics used in Orthopaedic surgery procedures. J Am Acad Orthop Surg. 2019;27(16):599–606.
9. Scheibler E, da Silva RM, Liite CE, Campos MW, Figueredo MA, Salum FG, Cherubini K. Stability and efficacy of combined nystatin and chlorhexidine against suspensions and biofilms of Candida albicans. Arch Oral Biol. 2018;89:70–6.
10. Hamill MB, Osato MS, Wilhelmus KR. Experimental evaluation of chlorhexidine gluconate for ocular antisepsis. Antimicrob Agents Chemother. 1984;26(6):793–6.
11. Oliveira Dos Santos C, Kolwijck E, van der Lee HA, Tehupeioy-Kooreman MC, Al-Hatmi AMS, Matayan E, Burton MJ, Eggink CA, Verweij PE. In Vitro activity of Chlorhexidine compared with seven antifungal agents against 98 Fusarium isolates recovered from fungal keratitis patients. Antimicrob Agents Chemother. 2019;63(8):e02669–18.
12. Xu Y, He Y, Zhou L, Gao C, Sun S, Wang X, Pang G. Effects of contact lens solution disinfectants against filamentous fungi. Optom Vis Sci. 2014;91(12):1440–5.
13. Rahman MR, Minassian DC, Sinivasan M, Martin ML, Johnson GJ. Trial of chlorhexidine gluconate for fungal corneal ulcers. Ophthalmoi Epidemiol. 1997;4(3):141–9.
14. Rahman MR, Johnson GJ, Husain R, Howlader SA, Minassian DC. Randomised trial of 0.2% chlorhexidine gluconate and 2.5% natamycin for fungal keratitis in Bangladesh. Br J Ophthalmol. 1998;82(8):919–25.
15. FlorCruz NV, Evans JR. Medical interventions for fungal keratitis. Cochrane Database Syst Rev. 2015;4:CD004241.
16. Boral H, van Diepeningen A, Erdem E, Yagmur M, de Hoog GS, Ilkit M, Meis JF, Al-Hatmi AMS. Mycotic keratitis caused by Fusarium solani sensu stricto (FSSCS), a case series. Mycopathologia. 2018;183(5):835–40.
17. Binder U, Maurer E, Lass-Flor G. Galleria mellonella: an invertebrate model to study pathogenecity in correctly defined fungal species. Fungal Biol. 2016;120(2):288–95.
18. Trevijano-Contador N, Zaragoza O. Immune response of Galleria mellonella against human fungal pathogens. J Fungi (Basel). 2018;5(1):3.
19. Telteira X, Araújo PV, Sirmiento RD, Cortés ME. Chlorhexidine: beta-cyclodextrin inhibits yeast growth by extraction of ergosterol. Braz J Microbiol. 2012;43(2):810–8.
20. Salah H, Al-Hatmi AM, Theelen B, Abukamar M, Hashim S, van Diepeningen AD, Lass-Flor C, Boelkout T, Altamolami M, Taj-Aldeen SJ. Phylogenetic diversity of human pathogenic Fusarium and emergence of uncommon virulent species. J Inf Secur. 2015;71(6):658–66.
21. John H. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi, approved standard. M38-A2. Clin Lab Stand Inst. 2008;28(16):1–35.
22. Odds FC. Synergy, antagonism, and what the checkerboard puts between them. J Antimicrob Chemother. 2003;52(1):1–1.

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