IN VITRO ACTIVITY OF CHLORHEXIDINE COMPARED WITH SEVEN ANTIFUNGAL AGENTS AGAINST 98 *FUSARIUM* ISOLATES RECOVERED FROM FUNGAL KERATITIS PATIENTS.

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Abstract

Background

Fungal keratitis is a common but severe eye infection in tropical and subtropical areas of the world. In regions with a temperate climate the frequency is rising in patients with contact lenses and following trauma. Early and adequate therapy is important to prevent disease progression and loss of vision. The management of *Fusarium* keratitis is complex, and the optimal treatment is not well defined. We investigated the *in vitro* activity of chlorhexidine and seven antifungal agents against a well characterized collection of *Fusarium* isolates, recovered from patients with *Fusarium* keratitis.

Patients and methods

The fungus culture collection of the Center of Expertise in Mycology Radboudumc/CWZ was searched for *Fusarium* isolates that were cultured from cornea scrapings, ocular biopsies, eye swabs and contact lens fluid containers from patients suspected of keratitis. The *Fusarium* isolates that were cultured from patients with confirmed keratitis were all identified using conventional and molecular techniques. Antifungal susceptibility testing was performed according to the EUCAST broth microdilution reference method. The antifungal agents tested included amphotericin B, voriconazole, posaconazole, miconazole, natamycin, 5-fluorocystosine, and caspofungin. In addition, the activity of chlorhexidine was determined.

Results

The fungal culture collection contained 98 *Fusarium* isolates of confirmed fungal keratitis cases from 83 Dutch patients and 15 Tanzanian patients. The isolates were collected between 2007 and 2017. *F. oxysporum* (n=24, 24.5%) was the most frequently isolated species followed by *F. solani sensu stricto* (n=18, 18.4%) and *F. petroliphilum* (n=11, 11.2%). *In vitro* amphotericin B was the most active antifungal drug followed by natamycin, voriconazole, posaconazole, and miconazole. Chlorhexidine showed activity against all. 5-Fluorocytosine showed no *in vitro* activity.

Conclusion

Amphotericin B showed the most favorable *in vitro* inhibition of *Fusarium* species followed by natamycin, voriconazole and chlorhexidine, while 5-fluorocytosine, posaconazole, miconazole and caspofungin showed no relevant inhibiting effect. However, chlorhexidine showed fungicidal activity against 90% of *F. oxysporum* strains and 100% of the *F. solani* strains.

Our study supports the clinical efficacy of chlorhexidine, and therefore warrants its further clinical evaluation for primary therapy of fungal keratitis, particularly in low and middle income countries where fungal keratitis is much more frequent and currently antifungal eye drops are often unavailable.

Introduction

Fungal keratitis is a common eye infection in tropical and subtropical areas of the world. In regions with a temperate climate fungal keratitis is uncommon, but mainly reported in patients with contact lenses and following trauma. Early therapy is important to prevent disease

67 progression or dissemination. A major complication of fungal keratitis is monocular
68 blindness, especially in tropical low and middle income countries (LMIC), where significant
69 delay in diagnosis and simply the unavailability of antifungals are common. Most cases of
70 fungal keratitis are caused by *Fusarium* species,[1,2] which are ubiquitous fast-growing
71 hyalohyphomycetes that are present in soil, water, and plants. The most common route of
72 infection is by (micro)trauma or disruptive ocular surface disease, as filamentous fungi are
73 unable to penetrate intact cornea.

74 The taxonomy of the *Fusarium* order is complex and still not well defined. Molecular
75 techniques have shown that the common medically relevant species, *F. solani* and *F.*
76 *oxysporum*, consist of multiple (sub)species.[3] Although, *in vitro* antifungal susceptibility
77 patterns of *Fusarium* species may vary greatly within each species, most species show high
78 minimal inhibitory concentrations (MICs) to the currently licensed antifungals.[4-7]. The
79 European Committee on Antimicrobial Susceptibility Testing (EUCAST) has not defined
80 epidemiological cut-off (ECOFF) values and clinical breakpoints for *Fusarium* species. In
81 2015 Espinel-Ingroff et al. published epidemiological cut-off values (ECVs) based on the
82 Clinical & Laboratory Standards Institute (CLSI) broth dilution method for antifungal
83 susceptibility testing (AFST).[8] As topical antifungal therapy is important for fungal keratitis
84 management, meaningful classification of isolates as resistant or susceptible is a challenge.
85 These factors complicate the management of *Fusarium* keratitis,[9] and the optimal treatment is
86 not well defined.

87 Currently, a 0.02% chlorhexidine solution is used for the treatment of *Acanthamoeba*
88 keratitis. A few studies have indicated that the disinfectant chlorhexidine might be an
89 effective, affordable and accessible treatment for fungal keratitis, which could benefit millions
90 of people who currently have no options.[10, 11] The meta-analysis in the Cochrane systematic
91 review written by FlorCruz and Evans show that chlorhexidine has a better clinical outcome

92 than natamycin and voriconazole.[12] To our knowledge, there are no published data describing
93 the MICs of chlorhexidine for *Fusarium* species. In this study, we investigated the *in vitro*
94 activity of chlorhexidine and seven antifungal agents against a molecularly characterized set
95 of *Fusarium* isolates, recovered from patients with keratitis.

96

97 **Results**

98 The fungal culture collection contained 98 *Fusarium* isolates from 83 patients with keratitis
99 from the Netherlands and 15 with keratitis from Tanzania. The isolates were collected
100 between 2007 and 2017. The first *Fusarium* isolate per patient was tested.

101 Molecular identification showed that *F. oxysporum* (n=24, 24.5%) was the most frequently
102 isolated species followed by *F. solani sensu stricto* (n=18, 18.4%) and *F. petroliphilum*
103 (n=11, 11.2%). Based on the assignment of the isolates to the according species complex, as
104 described by Salah et al.[3], the most frequently encountered complexes were *F. solani* species
105 complex (FSSC, n=43, 43.9%), *F. oxysporum* species complex (FOSC, n=24, 24.5%), *F.*
106 *fujikuroi* species complex (FFSC, n=16, 16.3%) and *F. dimerum* species complex (FDSC,
107 n=12, 12.2%). One isolate could not be assigned to any species complex or species and seems
108 to be a new *Fusarium* species.

109 The MIC distribution for the various species and species complexes are shown in Tables 1
110 and 2. *In vitro* amphotericin B was the most active antifungal drug followed by natamycin,
111 voriconazole, posaconazole, and miconazole. Chlorhexidine showed activity against all
112 species at a concentration of 8 to 32 mg/l, which corresponds with 1.56×10^{-3} to 6.25×10^{-3}%.
113 5-Fluorocytosine showed no *in vitro* activity.

114 Statistics. The median MIC and the MIC distributions of 5-fluorocytosine and caspofungin
115 showed no differences between any of the groups.

The MIC distributions of amphotericin B showed significant difference between the species complexes (Kruskal Wallis test, p = 0.002). The FDSC differed significant from FSSC and from FFSC.

For voriconazole the median MIC and the MIC distributions showed significant difference between the species complexes (p = 0.006 resp. p = 0.000, Kruskal Wallis test). The FSSC differed significant from FOSC, from FFSC and from FDSC.

The MIC distributions of posaconazole and miconazole showed significant difference between the species complexes (p = 0.000 resp. p = 0.001, Kruskal Wallis test). For posaconazole and miconazole the FFSC differed significant from FSSC and from FOSC.

The median MIC of natamycin was not different between the SC groups (p = 0.747, Kruskal-Wallis). On the other hand the MIC distributions of natamycin differed significant between the species complexes (p=0.015, Kruskal Wallis test). The FDSC differed significant from FSSC, from FOSC and from FFSC.

The median MIC and the MIC distributions of chlorhexidine showed significant difference between the species complexes (p = 0.000 resp. p = 0.000, Kruskal Wallis test). The FSSC differed significant from FOSC, from FFSC and from FDSC.

MIC values of posaconazole, miconazole, 5-fluorocytosine and caspofungin were high so determining the MFC of these agents was deemed clinically not relevant.

In vitro amphotericin B exhibited fungicidal effect on 60% of the *F. oxysporum* strains and 70% of the *F. solani* strains, the remainder of the strains showed a fungistatic effect (see figure 1). Natamycin was fungicidal against 80% of the *F. oxysporum* strains. However, in *F. solani* strains natamycin was mostly fungistatic, in only 30% it acted fungicidal. Voriconazole was fungicidal against 30% of the *F. oxysporum* strains and 50% of the *F. solani* strains.

Chlorhexidine showed fungicidal activity against 90% of *F. oxysporum* strains and 100% of the *F. solani* strains.

Discussion and conclusions

Chlorhexidine showed broad *in vitro* activity against all *Fusarium* species tested and compared with the antifungal agents showed the broadest fungicidal activity against the two species tested. Although it is likely that chlorhexidine is fungicidal in other *Fusarium* species, this was not tested. For chlorhexidine 95% of the 20 *Fusarium* isolates were killed at concentrations far below the 0.02% and 0.2% concentration, of which the 0.02% eye drops is already commonly used by ophthalmologists for treatment of *Acanthamoeba* keratitis. Another important advantage of chlorhexidine gluconate solution is the broad antimicrobial spectrum including Gram-positive and Gram-negative bacteria, lipid-enveloped viruses and *Acanthamoeba*.[12, 16]

A limited number of clinical trials have studied the effectiveness of chlorhexidine gluconate for the treatment of fungal keratitis. The aim of one trial was to find the most effective dose of chlorhexidine.[17] In comparison to the response with natamycin, the relative efficacy in a patient without prior antifungal treatment was 1.17 with chlorhexidine 0.05%, 1.43 with chlorhexidine 0.1% and 2.00 with chlorhexidine 0.2%. Their fungal isolates were not subjected to molecular identification and susceptibility testing. The second study of Rahman et al. showed a relative efficacy of 1.85 (CI 1.01-3.39, p = 0.04) with chlorhexidine 0.2% in comparison to natamycin. Of the non-severe ulcers 66.7% was healed at day 21 with chlorhexidine and 36.0% with natamycin. However, this trial was not double blinded due to the fact that personnel could identify the selected treatment because the solutions of chlorhexidine and natamycin were visibly different.[10] The susceptibility testing was

performed with a non-reference well diffusion method (0.2% chlorhexidine, 2.5% natamycin, 1% econazole). The concentration of natamycin used was 2.5%, which is half the current standard therapeutic concentration of 5%. In addition, the method of identification of the strains was also not mentioned.

In the Netherlands, the available antifungal agents, which can be used as eye drops are amphotericin B 0.15% (1,500 mg/L), voriconazole 1% (10.000 mg/L) and the disinfectant chlorhexidine 0.02% (200 mg/L). These formulas are not commercially available but are prepared by hospital pharmacists on request. In other countries natamycin (5% suspension; 50.000 mg/L) is available and frequently used in the setting of fungal keratitis.[2, 10-12, 18-23] These concentrations exceed by far the *in vitro* determined MICs of the *Fusarium* isolates (tables 1 and 2). However, effectiveness depends on many factors including the ability of the compound to penetrate ocular tissues, local bioavailability, and drug toxicity.

The most important route of penetration of topical antifungals into ocular tissue is through the cornea, mostly by diffusion. The polyenes, amphotericin B and natamycin, are compounds with a high molecular mass (i.e. > 500 Da) and as a consequence barely penetrate intact cornea epithelium.[24] This leads to the necessity of regularly performing abrasions of the cornea during treatment with amphotericin B. In high doses, amphotericin B can be toxic to the cornea but the 0.15% solution is well tolerated.[24] Due to the viscous nature of natamycin suspension and its poor penetration it is only suitable for treatment of superficially located keratomycosis. Furthermore, compounds that are lipophilic can penetrate across the corneal stroma, while hydrophilic agents are able to penetrate all the layers of the cornea.

There is little known about the corneal penetration of the cationic antisepticum chlorhexidine gluconate. In a small animal study Vontobel et al. showed that the compound did not penetrate through the intact or mechanically damaged cornea into the anterior chamber.[25] It

187 seems that chlorhexidine accumulates within the cornea, explaining the need to treat deep
188 seeted *Acanthamoeba* for a long time.

189 Amphotericin B showed the most favorable *in vitro* inhibition of *Fusarium* species followed
190 by natamycin, voriconazole and chlorhexidine, while 5-fluorocytosine, posaconazole,
191 miconazole and caspofungin showed no relevant inhibiting effect. However, chlorhexidine
192 showed fungicidal activity against 90% of *F. oxysporum* strains and 100% of the *F. solani*
193 strains.

194 The differences in AFST between isolates belonging to the same species complex justifies
195 conducting molecular identification to species complex level. In general, the species
196 belonging to the FDSC and the FFSC are more susceptible to chlorhexidine, amphotericin B,
197 natamycin, voriconazole and posaconazole (only FFSC). These differences cannot be
198 predicted by identification based on conventional methods because the characteristics of
199 morphology and microscopy are not species specific.

200 Our study supports the clinical efficacy of chlorhexidine, and therefore warrants its further
201 clinical evaluation for primary therapy of fungal keratitis, particularly in LMIC where fungal
202 keratitis is much more frequent and currently antifungal eye drops are often unavailable.
203 Further studies should investigate the *in vitro* interaction of chlorhexidine with antifungal
204 agents to support alternate administration and combination therapy.

205

206 **Patients and methods**

207 The fungus culture collection of the Center of Expertise in Mycology Radboudumc/CWZ was
208 searched for *Fusarium* isolates that were cultured from cornea scrapings, ocular biopsies, eye
209 swabs and contact lens fluid containers from patients suspected of keratitis. All isolates had

been identified up to genus level using conventional techniques. For accurate species identification, sequencing of the TEF1 gene was performed.[3]

Antifungal susceptibility testing was performed according to the EUCAST broth microdilution reference method.[13, 14] The antifungal agents tested included amphotericin B (Bristol Myers Squibb), voriconazole (Pfizer), posaconazole (Merck & Co), miconazole (Janssen Cilag), natamycin (Sigma-Aldrich), 5-fluorocystosine (Hoffman la Roche), and caspofungin (Merck & Co). In addition, the activity of chlorhexidine (Pharmaline) was determined. The test range of the antifungal agents was 0.02 – 16 mg/L for amphotericin B, voriconazole, posaconazole, miconazole, caspofungin and natamycin, and 0.03 – 32 mg/L for 5-fluorocytosine. For chlorhexidine a concentration range of 1 to 1024 mg/L was used which corresponds with 0.000195% to 0.2%. All antifungal agents and chlorhexidine were dissolved in RPMI 1640 supplemented with glucose to a final concentration of 2%. The MICs were determined with an inverted mirror after 48 hours at 35°C as the lowest drug concentration with complete inhibition of growth visible by eye for amphotericin B, voriconazole, posaconazole, miconazole, natamycin, 5-fluorocystosine and chlorhexidine. The endpoint for echinocandins was the minimal effective concentration (MEC). The MEC for caspofungin was determined with an inverted microscope after 48 hours at 35°C as the lowest drug concentration in which abnormal, short, and branched hyphal clusters are observed in contrast to the long, unbranched, elegant hyphal elements that are visible in the growth control well. *Aspergillus fumigatus* ATCC 204305 and *Aspergillus flavus* ATCC 204304 were used as quality control strains as recommended by the EUCAST.[13]

Ten *F. oxysporum* and 10 *F. solani* were used to determine the minimal fungicidal concentration (MFC) for the antifungal agents and chlorhexidine. After reading the MICs at 48 h, the 96-wells plates were shaken to loosen the fungal elements. Thereafter, 20µl of all the wells with no visible growth and 20µl of the growth control were plated on Sabouraud agar

(Oxoid). The plates were incubated for 24 and 48 hours at 35°C. The MFC was determined as the lowest drug concentration, which leads to 99-99.5% growth inhibition compared to the growth control. Antifungal agents were considered fungicidal when the MIC value was no more than two dilution steps lower than the MFC.[15] If the difference between MIC and MFC was > 2 dilution steps the antifungal agent was classified as fungistatic.[15]

Statistical analysis was performed with IBM SPSS Statistics 24. *In vitro* susceptibility differences between *Fusarium* species and differences between species complexes were tested with a non-parametric test (one-way ANOVA, Kruskal-Wallis). A p-value of <0.05 was determined as significant. In order to correct for multi-testing in the search for which group(s) differed from each other the p-value was adjusted according to the Bonferroni correction method (e.g. significance level [<0.05] divided by the number of tests needed). The Bonferonni correction for the species groups was $p < 0.0011$, for the species complex groups the Bonferonni was $p < 0.0083$. Species and species complexes with only one isolate where not taken into account in the statistical analysis. For every antifungal or antiseptic agent we tested for significant differences between the species complexes by comparing the median MIC or percentage of the concentration and comparing the distributions between the groups. After this comparison the groups which were responsible for the significant difference were determined through comparing one group to another group with the Mann-Whitney U test.

The collection of samples from Dutch participants were collected during the routine standard of care. Therefore, we didn't need their informed consent in accordance to the Dutch Ethics Committee of the Radboud University Medical Center.

The collection of samples from Tanzanian participants was approved by the Ethics Committees of the National Institute for Medical Research, Tanzania and the London School

of Hygiene & Tropical Medicine, United Kingdom. Informed consent was obtained from all participants.

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Contributions of the authors:

COdS performed susceptibility testing, collected the data and drafted the manuscript. EK reviewed the manuscript from the microbiological point of view. HL and MTK performed susceptibility testing and reviewed the manuscript from the microbiological point of view. AAH performed the molecular identification of the *Fusarium* strains and reviewed the manuscript. EM and MB collected the Fusarium strains from Tanzania and reviewed the manuscript from the clinical point of view. CE reviewed the manuscript from the clinical point of view. PV reviewed the manuscript from the microbiological point of view and helped with editing the manuscript. All authors have read and approved the final and submitted manuscript.

Legends

Figure 1. The proportion of fungicidal and fungistatic *in vitro* effect of amphotericin B, natamycin, voriconazole and the disinfectant chlorhexidine depicted of *Fusarium oxysporum* (n=10) and *Fusarium solani* (n=10), all of which were isolated from patients with fungal keratitis. Blue is fungicidal (upper proportion of the bars) and green is fungistatic proportion.

References

1. **Iyer S, Tuli S, Wagoner R.** 2006. Fungal Keratitis: Emerging Trends and Treatment Outcomes. Eye & Contact Lens: Science & Clinical Practice **32:**267-271.

2. **Shukl, PK, Kumar M, Keshava GBS.** 2008. Mycotic keratitis: an overview of diagnosis and therapy. Mycoses **51:**183-199.

3. **Salah H, Al-Hatmi AMS, Theelen B, Abukamar M, Hashim S, van Diepeningen AD, Lass-Florl C, Boekhout T, Almaslamani M, Taj-Aldeen SJ.** 2015. Phylogenetic diversity of human pathogenic Fusarium and emergence of uncommon virulent species. J Infect **71:**658-66.

4. **Al-Hatmi AM, van Diepeningen AD, Curfs-Breuker I, de Hoog GS, Meis JFGM.** 2015. Specific antifungal susceptibility profiles of opportunists in the Fusarium fujikuroi complex. J Antimicrob Chemother **70:**1068-1071.

5. **Alastruey-Izquierdo A, Cuenca-Estrella M, Monzón A, Mellado E, Rodríguez-Tudela JL.** 2008. Antifungal susceptibility profile of clinical Fusarium spp. isolates identified by molecular methods. J Antimicrob Chemother **61:**805-809.

6. **Araujo R, Oliveira M, Amorim A, Sampaio-Maia B.** 2015. Unpredictable susceptibility of emerging clinical moulds to tri-azoles: review of the literature and upcoming challenges for mould identification. Eur J Clin Microbiol Infect Dis. **34:**1289-1301.

7. **Tortorano AM, Prigitano A, Dho G, Esposto MC, Gianni C, Grancini A, Ossi C, Viviani MA.** 2008. Species Distribution and In Vitro Antifungal Susceptibility Patterns of 75 Clinical Isolates of Fusarium spp. from Northern Italy. Antimicrob Agents Chemother **52:**2683-2685.

8. **Espinel-Ingroff A, Colombo AL, Cordoba S, Dufresne PJ, Fuller J, Ghannoum M, Gonzalez GM, Guarro J, Kidd SE, Meis JF, Melhem TMSC, Pelaez T, Pfaller MA, Szeszs MW, Takahaschi JP, Tortorano AM, Wiederhold NP, Turnidge J.** 2016. International Evaluation of MIC Distributions and Epidemiological Cutoff Value (ECV) Definitions for Fusarium Species Identified by Molecular Methods for the CLSI Broth Microdilution Method. Antimicrob Agents Chemother **60:**1079-1084.

9. **Guarro J.** 2013. Fusariosis, a complex infection caused by a high diversity of fungal species refractory to treatment. Eur J Clin Microbiol Infect Dis **32:**1491.

10. **Rahman MR, Johnson GJ, Husain R, Howlader SA, Minassian DC.** 1998. Randomised trial of 0.2% chlorhexidine gluconate and 2.5% natamycin for fungal keratitis in Bangladesh. Br J Ophthalmol **82:**919-925.

11. **Qiu S, Zhao GQ, Lin J, Wang X, Hu LT, Du ZD, Wang Q, Zhu CC.** 2015. Natamycin in the treatment of fungal keratitis : a systematic review and Meta-analysis. Int J Ophtalmol. **8:**597-602.

12. **FlorCruz NV, Evans JR.** 2015. Medical interventions for fungal keratitis. Cochrane Database of Systematic Reviews 2015, Issue 4. Art. No.: CD004241. https://www.cochrane.org/CD004241/EYES_medical-treatments-for-fungal-infection-of-the-cornea-clear-front-part-of-the-eye.

13. **Arendrup MC, Meletiadis J, Mouton JW, Lagrou K, Hamal P, Guinea J, Subcommittee on AFST of the ESCMID EUCAST.** 2017. EUCAST DEFINITIVE DOCUMENT E.DEF 9.3.1 Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_9_3_1_Mould_testing__definitive.pdf.

14. **Roiquez Tudela JL, Donnelly JP, Arenup MC, Arikan S, Barchiesi F, Bille J, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Denning DW, Fegeler W, Gaustad P, Lass-Florl C, Moore C, Richardson M, Schmalreck A, Velegraki JA, Verweij PE.** 2008. EUCAST Technical Note on the method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia-forming moulds. Clin Microbiol and Infect **14:**982-984.

15. **Graybill J, Burgess D, Hardin T.** 1997. Key issues concerning fungistatic versus fungicidal drugs. Eur J Clin Microbiol Infect Dis **16:**42-50.

16. **McDonnell G, Russell AD.** 1999. Antiseptics and Disinfectants: Activity, Action, and Resistance. Clin Microbiol Rev **12:**147-179.

17. **Rahman MR, Minassian DC, Srinivasan M, Martin MJ, Johnson GJ.** 1997. Trial of chlorhexidine gluconate for fungal corneal ulcers. Ophtalmic Epidemiol **4:**141-149.

18. **Jones DB, Forster FK, Rebell G.** 1972. *Fusarium solani* keratitis treated with natamycin (pimaricin): eighteen consecutive cases. Arch Ophthalmol **88:**147-154.

342 19. **Prajna NV, John RK, Nirmalan PK, Lalitha P, Srinivasan M.** 2003. A randomised
343 clinical trial comparing 2% econazole and 5% natamycin for the treatment of fungal keratitis.
344 Br J Ophthalmol **87:**1235-1237.

345 20. **Prajna NV, Krishnan T, Mascarenhas J, Rajaraman R, Prajna L, Srinivasan M,**
346 **Raghavan A, Oldenburg CE, Ray KJ, Zegans ME, McLeod SD, Porco TC, Acharya NR,**
347 **Lietman TM.** 2013. The Mycotic Ulcer Treatment Trial: A Randomized Trial Comparing
348 Natamycin vs Voriconazole. JAMA Ophthalmol **131:**422-429.

349 21. **Prajna NV, Mascarenhas J, Krishnan T, Reddy PR, Prajna L, Srinivasan M,**
350 **Vaitilingam CM, Hong KC, Lee SM, McLeod SD, Zegans ME, Porco TC, Lietman TM,**
351 **Acharya NR.** 2010. Comparison of Natamycin and Voriconazole for the Treatment of Fungal
352 Keratitis. Arch Ophthalmol **128:**672-678.

353 22. **Sun CQ, Lalitha P, Prajna NV, Karpagam R, Geetha M, O'Brien KS, Oldenburg**
354 **CE, Ray KJ, McLeod SD, Acharya NR, Lietman TM.** 2014. Association between in vitro
355 susceptibility to natamycin and voriconazole and clinical outcomes in fungal keratitis.
356 Ophthalmology. **121:**1500.e1.

357 23. **Thomas PA, Kaliamurthy J.** 2013. Mycotic keratitis: epidemiology, diagnosis and
358 management. Clin Microbiol Infect **19:**210-220.

359 24. **Kaur IP, Kakkar S.** 2010. Topical delivery of antifungal agents. Expert Opin Drug Deliv
360 **7:**1303-1327.

361 25. **Vontobel SF, Abad-Villar EM, Kaufmann C, Zinkernagel AS, Hauser PC, Thiel MA.**
362 2015. Corneal Penetration of Polyhexamethylene Biguanide and Chlorhexidine Digluconate. J
363 Clin Exp Ophthalmol 6: 430

Table 1. Molecularly identified fusarial keratitis isolates and their susceptibility profile to eight antifungal agents including chlorhexidin and natamycin.

	MIC* % [median (range)]	MIC* mg/L [median (range)]							MEC* mg/L [median (range)]
***Fusarium* species (n)**	**CHX**	**CHX**	**AMB**	**VCZ**	**5-FC**	**MCZ**	**NAT**	**POS**	**CAS**
F. species (1)	0.003	16	0.5	2	32	16	8	16	16
F. falciforme (7)	0.006 (0.002-0.006)	32 (8-32)	2 (1-8)	16 (8-16)	>32 (>32)	16 (16)	8 (8-16)	16 (16)	16 (16)
F. keratinoplasticum (7)	0.003 (0.002-0.006)	16 (8-32)	4 (2-4)	8 (4-16)	>32 (>32)	16 (16)	4 (4-8)	16 (16)	16 (0.5-16)
F. petroliphilum (11)	0.003 (0.002-0.006)	16 (8-32)	2 (0.5-4)	8 (4-16)	>32 (>32)	16 (16)	4 (4-8)	16 (16)	16 (2-16)
F. solani (18)	0.006 (0.002-0.006)	32 (8-32)	2 (0.063-16)	8 (4-16)	>32 (>32)	16 (8-16)	8 (4-16)	16 (8-16)	16 (4-32)
F. oxysporum (24)	0.002 (0.0002-0.012)	8 (1-64)	2 (0,25-16)	4 (2-16)	32 (0.063-32)	16 (16)	8 (4-8)	16 (16)	16 (0.063-32)
F. musae (1)	0.003	16	2	4	>32	8	8	1	16
F. verticillioides (3)	0.003 (0.001-0.003)	16 (4-16)	2 (1-8)	2 (1-2)	>32 (>32)	1 (0.25-8)	8 (2-8)	0.5 (0.25-1)	16 (16)
F. proliferatum (7)	0.002 (0.001-0.012)	8 (4-64)	2 (1-4)	4 (2-8)	>32 (>32)	16 (16)	8 (4-8)	4 (2-16)	16 (16)
F. ramigenum (1)	0.003	16	4	1	>32	16	4	1	16
F. sacchari (1)	0.002	8	2	1	>32	4	8	0.25	16
F. lactis (3)	0.002 (0.002-0.003)	8 (8-16)	2 (0.5-4)	4 (2-8)	>32 (>32)	16 (16)	8 (8)	16 (2-16)	16 (16)
F. equiseti (1)	†	†	1	8	†	†	†	16	32
F. dimerum (8)	0.002 (0.002-0.003)	8 (8-16)	1 (0.5-2)	8 (4-8)	>32 (>32)	16 (16)	4 (4-16)	16 (16)	16 (2-16)
F. delphinoides (4)	0.001 (0.001-0.002)	4 (4-8)	0.5 (0.125-1)	2 (2)	>32 (>32)	16 (16)	4 (2-4)	8 (1-16)	16 (2-16)
F. ambrosium (1)	0.006	32	2	16	>32	16	8	16	1

*MIC, minimal inhibitory concentration; MEC, minimal effective concentration.

*AMB, amphotericin B; VCZ, voriconazole; POS, posaconazole; MCZ, miconazole; NAT, natamycin; 5FC, 5-fluorocytosin; CHLX, chlorhexidine; CAS, caspofungin.

†susceptibility testing for this antifungal agent was not performed.

Table 2. Fusarial keratitis isolates assigned according to the species complex and their susceptibility profile to eight antifungal agents including chlorhexidin and natamycin.

	MIC* % [median (range)]	MIC* mg/L [median (range)]							MEC* mg/L [median (range)]
***Fusarium* species complex (n)**	**CHX**	**CHX**	**AMB**	**VCZ**	**5-FC**	**MCZ**	**NAT**	**POS**	**CAS**
Unknown (1)	0.003	16	0.5	2	32	16	8	16	16
F. solani species complex - FSSC (43)	0.003 (0.002-0.006)$^{\delta}$	16 (8-32)	2 (0.063-16)	8 (4-16)$^{\delta}$	>32 (>32)	16 (8-16)	8 (4-16)	16 (8-16)	16 (0.5-32)
F. oxysporum species complex - FOSC (24)	0.002 (0.002-0.012)	8 (2-64)	2 (0.25-16)	4 (2-16)	32 (0.063-32)	16 (16)	8 (4-8)	16 (16)	16 (0.063-32)
F. fujikuroi species complex - FFSC (16)	0.002 (0.001-0.012)	8 (4-64)	2 (0.5-8)	4 (1-8)	>32 (>32)	16 (0.25-16)$^{\delta}$	8 (2-8)	2 (0.25-16)$^{\delta}$	16 (16)
F. incarnatus-equiseti species complex - FIESC (1)	†	†	1	8	†	†	†	16	32
F. dimerum species complex - FDSC (12)	0.002 (0.0008-0.003)	8 (4-16)	1 (0.125-2)$^{\delta}$	8 (2-8)	>32 (>32)	16 (16)	4 (2-16)$^{\delta}$	16 (1-16)	16 (2-16)
Ambrosia *Fusarium* Clade - AFC (1)	0.006	32	2	16	>32	16	8	16	1

*MIC, minimal inhibitory concentration; MEC, minimal effective concentration.

*AMB, amphotericin B; VCZ, voriconazole; POS, posaconazole; MCZ, miconazole; NAT, natamycin; 5FC, 5-fluorocytosin; CHLX, chlorhexidine; CAS, caspofungin.

†susceptibility testing for this antifungal agent was not performed.

δSignificant difference of the median and or distribution range between the groups of species complex.