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Antifungal effect of ophthalmic preservatives phenylmercuric nitrate and benzalkonium chloride on ocular pathogenic filamentous fungi

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ABSTRACT

In the present study, the antifungal effects of phenylmercuric nitrate and benzalkonium chloride versus those of natamycin and ketoconazole were assessed against 216 filamentous fungi isolates from cases of fungal keratitis. They included 112 *Fusarium* isolates, 94 *Aspergillus* isolates, and 10 *Alternaria alternata* isolates. The strains were tested by broth dilution antifungal susceptibility testing of filamentous fungi approved by the Clinical and Laboratory Standards Institute M38-A document. The results showed that the MIC_{50} values of phenylmercuric nitrate were 0.0156, 0.0156, and 0.0313 μg/mL for *Fusarium* spp., *Aspergillus* spp., and *A. alternata*, respectively. The MIC_{90} values of phenylmercuric nitrate were 0.0313, 0.0313, and 0.0313 μg/mL for *Fusarium* spp., *Aspergillus* spp., and *A. alternata*, respectively. The MIC_{50} values of benzalkonium chloride were 16, 32, and 8 μg/mL for *Fusarium* spp., *Aspergillus* spp., and *A. alternata*, respectively. The MIC_{90} values of benzalkonium chloride were 32, 32, and 16 μg/mL for *Fusarium* spp., *Aspergillus* spp., and *A. alternata*, respectively. The study indicates that phenylmercuric nitrate has considerable antifungal activity and its effect is significantly superior to those of benzalkonium chloride, natamycin, and ketoconazole against ocular pathogenic filamentous fungi in vitro, deserving further investigation for treating fungal keratitis as a main drug.

1. Introduction

Fungal keratitis is the most common and severe infectious corneal diseases in many developing countries. The disease has become the leading cause of corneal blindness in China because of higher incidence and the unavailability of effective antifungal agents (Sun et al., 2007; Xie et al., 2001; Zhong et al., 2007). Fungal keratitis may constitute 46.7–61.9% of all cases of suppurative keratitis inpatients, and 35.1% of all cases of infectious keratitis underwent penetrating keratoplasty in some regions in China (Xie et al., 2001; Zhong et al., 2007). Filamentous fungi, mainly *Fusarium* spp. (65–77.6%) and *Aspergillus* spp. (10.8–20.5%), are most commonly associated with fungal keratitis in China (Sun et al., 2007; Xie et al., 2001, 2008; Zhong et al., 2007). Fungal keratitis still remains a therapeutic challenge for the ophthalmologist. To date, only fluconazole and natamycin are commercially available for fungal keratitis in China. Fluconazole is inactive against ocular pathogenic filamentous fungi (Rao et al., 1997; Xu et al., 2009a, 2010b). Natamycin is the only commercially available topical antifungal preparation approved by the Food and Drug Administration for ophthalmic use (Srinivasan, 2004; Thomas, 2003). It is insoluble in water and is stable in 5% suspension. After topical application, natamycin penetrates the cornea and conjunctiva poorly, effective drug levels are not achieved in either the cornea or aqueous (O'Day et al., 1986), and it is therefore useful only in the treatment of superficial infections that are not severe. Natamycin is the drug of choice for therapy of fungal keratitis in many countries particularly for keratitis due to filamentous fungi (Pradhan et al., 2011; Srinivasan, 2004; Thomas, 2003). Some studies, however, have shown that *Aspergillus* spp. are resistant to natamycin and poor outcomes with natamycin for patients with fungal keratitis (Lalitha et al., 2007; Mehta et al., 2008; Pradhan et al., 2011; Xu et al., 2009b, 2010a,b). The therapy of fungal keratitis is restricted by the relative unavailability of effective antifungal agents. Corneal lesions fail to resolve in many patients who receive antifungal treatment and some patients get marked loss of vision and eventually complete perforation of the cornea, ultimately requiring penetrating keratoplasty, and even enucleation or evisceration (Pradhan et al., 2011; Srinivasan, 2004; Thomas, 2003; Xie and Zhai, 2005; Xie et al., 2001). Therefore, it is very important and urgent to develop new antifungal agents that are active against ocular pathogenic filamentous fungi and may have a role in future studies of antifungal eye drops.

Phenylmercuric nitrate and benzalkonium chloride are preservatives commonly used in topical ophthalmic preparations because of

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their bactericidal property. However, the efficacy of phenylmercuric nitrate and benzalkonium chloride against ocular pathogenic fungi has not been evaluated so far. This study reports on the antifungal effects of phenylmercuric nitrate and benzalkonium chloride in comparison with those of natamycin and ketoconazole against ocular pathogenic filamentous fungi in vitro.

2. Materials and methods

2.1. Antifungal agents

Phenylmercuric nitrate (Taixing Chemical, Jiangsu, China), benzalkonium chloride (Sigma-Aldrich, St. Louis, MO, USA), natamycin (Yinxiang Biotechnology, Zhejiang, China), and ketoconazole (Nanjing Second Pharmaceutical Factory, Nanjing, China) were studied. They were dissolved in 100% dimethyl sulfoxide, respectively. The stock solutions were prepared at concentration of 400 μg/mL for phenylmercuric nitrate and 1600 μg/mL for benzalkonium chloride, natamycin, and ketoconazole. Drug dilutions were made in RPMI 1640 medium buffered to pH 7.0 with 0.165 mol/L of morpholinepropanesulfonic acid. Final concentrations ranged from 0.0078 to 4 μg/mL for phenylmercuric nitrate and from 0.0313 to 16 μg/mL for benzalkonium chloride, natamycin, and ketoconazole.

2.2. Test isolates

A total of 216 strains of fungi isolated from patients with fungal keratitis from Henan Eye Institute in Zhengzhou, China, were investigated. These isolates were identified to the species level based on morphology by standard methods (Wang, 2005; Wu, 2005). They included 112 *Fusarium* isolates (82 *Fusarium solani* species complex, 20 *Fusarium verticillioides* species complex, and 10 *Fusarium oxysporum* species complex), 94 *Aspergillus* isolates (61 *Aspergillus flavus* species complex, 11 *Aspergillus fumigatus* species complex, 12 *Aspergillus versicolor* species complex, and 10 *Aspergillus niger* species complex), and 10 *Alternaria alternata* isolates.

2.3. Antifungal-susceptibility tests

Inocula were prepared according to Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) document M38-A (NCCLS, 2002). Simply, the isolates were passaged twice at an interval of 7 days on potato dextrose agar slants at 35 °C for 7 days *(Aspergillus* spp.) or at 35 °C for 72 h, then at 26 °C until day 7 (*Fusarium* spp. and *A. alternata*). Seven-day-old colony was covered with 1 mL of sterile 0.85% saline, and then suspension was made. The resulting mixture of conidia and hyphal fragments was withdrawn. After heavy particles were allowed to settle for 3 to 5 min, the upper homogeneous suspension was collected and mixed with a vortex mixer for 15 s. The turbidity of the supernatants was measured spectrophotometrically at a wavelength of 530 nm, and transmission was adjusted to 68% to 70% (*Fusarium* spp. and *A. alternata*) or to 80% to 82% (*Aspergillus* spp.). These suspensions were diluted 1:50 in RPMI 1640. The 1:50 inoculum dilutions corresponded to twice the density needed of approximately 0.4×10^4 to 5×10^4 CFU/mL.

A broth microdilution method was performed by following the CLSI M38-A document. The tests were performed in duplicate in 96-well flat-bottom microtitration plates. Each well was inoculated with 0.1 mL of the 2× conidial inoculum suspension. The growth control wells contained 0.1 mL of the corresponding diluted inoculum suspension and 0.1 mL of RPMI-1640 broth without antifungal agents (with dimethyl sulfoxide 2%). *Candida parapsilosis* ATCC22019 was used as quality control. Quality control was tested in the same manner and was included in each test. Plates were incubated at 35 °C for 48 h.

MIC was determined as the lowest concentration of phenylmercuric nitrate, benzalkonium chloride, or natamycin that prevented any discernible growth or of ketoconazole that prevented an approximately 50% discernible growth compared to the growth of the control. The MIC range and mode, the MIC_{50} value, and the MIC_{90} value were provided for the isolates using the SPSS statistical package (version 17.0, SPSS, Chicago, IL, USA). For calculation, any high off-scale MIC was converted to the next higher concentration.

3. Results

All the isolates produced detectable growth after 48 h of incubation at 35 °C. The MIC of the quality control strain was within the reference ranges for each test. The in vitro activities of phenylmercuric nitrate, benzalkonium chloride, natamycin, and ketoconazole against the isolates are summarized in Table 1.

The MIC_{90} values of phenylmercuric nitrate were 0.0313, 0.0313, and 0.0313 μg/mL for *Fusarium* spp., *Aspergillus* spp., and *A. alternata*, respectively. The MIC_{90} values of benzalkonium chloride were 32, 32,

Table 1
In vitro susceptibilities of ocular *Fusarium, Aspergillus*, and *Alternaria alternata* isolates to phenylmercuric nitrate, benzalkonium chloride, natamycin, and ketoconazole.

Organism (no.) and antifungal agents	MIC values (μg/mL)			
	Range	Mode	MIC_{50}	MIC_{90}
Fusarium solani species complex (82)				
Phenylmercuric nitrate	0.0078–0.0625	0.0078	0.0156	0.0313
Benzalkonium chloride	8–32	16	16	32
Natamycin	4–32	4	4	8
Ketoconazole	1–32	32	32	32
Fusarium verticillioides species complex (20)				
Phenylmercuric nitrate	0.0078–0.0313	0.0156	0.0156	0.0313
Benzalkonium chloride	8–32	16	16	16
Natamycin	4–8	4	4	8
Ketoconazole	1–32	2	4	32
Fusarium oxysporum species complex (10)				
Phenylmercuric nitrate	0.0078–0.0313	0.0078	0.0078	0.0156
Benzalkonium chloride	8–16	16	16	16
Natamycin	4–8	4	4	8
Ketoconazole	2–32	32	8	32
Fusarium spp. (112)				
Phenylmercuric nitrate	0.0078–0.0625	0.0078	0.0156	0.0313
Benzalkonium chloride	8–32	16	16	32
Natamycin	4–32	4	4	8
Ketoconazole	1–32	32	32	32
Aspergillus flavus species complex (61)				
Phenylmercuric nitrate	0.0039–0.0313	0.0156	0.0156	0.0313
Benzalkonium chloride	4–32	32	32	32
Natamycin	4–32	32	32	32
Ketoconazole	0.25–16	1	1	1
Aspergillus fumigatus species complex (11)				
Phenylmercuric nitrate	0.0156–0.0313	0.0313	0.0313	0.0313
Benzalkonium chloride	4–16	8	8	16
Natamycin	4–32	4	4	4
Ketoconazole	1–2	2	2	2
Aspergillus versicolor species complex (12)				
Phenylmercuric nitrate	0.0078–0.0313	0.0078	0.0078	0.0156
Benzalkonium chloride	4–32	8	8	32
Natamycin	4–32	8	8	32
Ketoconazole	0.25–1	1	0.5	1
Aspergillus niger species complex (10)				
Phenylmercuric nitrate	0.0078–0.0313	0.0078	0.0078	0.0156
Benzalkonium chloride	2–8	4	4	4
Natamycin	0.25–16	4	4	8
Ketoconazole	0.0625–2	1	1	2
Aspergillus spp. (94)				
Phenylmercuric nitrate	0.0039–0.0313	0.0156	0.0156	0.0313
Benzalkonium chloride	2–32	32	32	32
Natamycin	0·.25–32	32	32	32
Ketoconazole	0.0625–16	1	1	2
Alternaria alternata (10)				
Phenylmercuric nitrate	0.0078–0.0313	0.0313	0.0313	0.0313
Benzalkonium chloride	2–16	8	8	16
Natamycin	2–8	4	4	4
Ketoconazole	0.0625–4	0.5	0.5	2