In Vitro Activity of Thimerosal against Ocular Pathogenic Fungi[▽]

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The *in vitro* activity of thimerosal versus those of amphotericin B and natamycin was assessed against 244 ocular fungal isolates. The activity of thimerosal against *Fusarium* spp., *Aspergillus* spp., and *Alternaria alternata* was 256 times, 512 times, and 128 times, respectively, greater than that of natamycin and 64 times, 32 times, and 32 times, respectively, greater than that of amphotericin B. Thimerosal's antifungal activity was significantly superior to those of amphotericin B and natamycin against ocular pathogenic fungi *in vitro*.

The problem of keratomycosis in developing countries like China is more acute because of its higher incidence and the unavailability of effective antifungals (18, 28, 30). To date, only fluconazole and natamycin are commercially available for ocular use in China. Fluconazole has high bioavailability against *Candida* spp., but *Fusarium* spp. and *Aspergillus* spp. are resistant to it (3, 27, 29). *Fusarium* spp. and *Aspergillus* spp. are more commonly associated with keratomycosis, while *Candida* spp. are rarely implicated as etiological agents of keratomycosis in China (23, 26). Natamycin is the only topical ophthalmic antifungal compound approved in the United States (14). Natamycin is poorly soluble in water. After topical application, natamycin penetrates the cornea and conjunctiva poorly and effective drug levels are not achieved in either the cornea or the aqueous humor (15); it is therefore useful only in the treatment of superficial keratomycosis. Due to the relative unavailability of effective antifungals, keratomycosis fails to resolve in many of the patients who receive antifungal treatment; some patients experience vision loss and eventually corneal perforation, ultimately require penetrating keratoplasty, or even enucleation or evisceration (20, 28). Therefore, it is very important and urgent to explore broad-spectrum antifungals to effectively suppress a wide variety of ocular fungal pathogens and to develop new antifungal eye drops to combat this vision-threatening infection.

Thimerosal is a preservative commonly used in ophthalmic solutions, otic drops, topical medicine, and vaccines because of its bactericidal property. However, the efficacy of thimerosal against ocular pathogenic fungi has not been evaluated so far. The present study was performed to determine the antifungal activity of thimerosal versus those of amphotericin B and natamycin against ocular pathogenic fungi *in vitro*. To our

knowledge, this is the first study to determine the antifungal activity of thimerosal against main ocular pathogenic fungi. Results obtained in this study may contribute to the development of new antifungal eye drops.

Two hundred forty-four strains of fungi isolated from patients with keratomycosis from the Henan Eye Institute in Zhengzhou, China, were investigated. These isolates were identified based on morphology by standard methods (22, 23, 25). They included 136 *Fusarium* isolates, 98 *Aspergillus* isolates, and 10 *Alternaria alternata* isolates. *Candida parapsilosis* ATCC 22019 was used as quality control for each test.

Thimerosal (Yili Pharmaceutical Co. Ltd., Beijing, China), amphotericin B (Bristol-Myers Squibb, Princeton, NJ), and natamycin (Yinxiang Biotechnology Co. Ltd., Zhejiang, China) were studied. They were all dissolved in 100% dimethyl sulfoxide. The stock solutions were prepared at concentrations of 400 µg/ml for thimerosal and 1,600 µg/ml for amphotericin B and natamycin. Drug dilutions were made in RPMI 1640 medium buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid. Final concentrations ranged from 0.0078 to 4 µg/ml for thimerosal and from 0.0313 to 16 µg/ml for amphotericin B and natamycin.

A broth microdilution method was performed by following the Clinical and Laboratory Standards Institute M38-A2 document (13). The final inoculum was 0.4×10^4 to 5×10^4 CFU/ml. Following incubation at 35°C for 48 h, the MIC was determined as the lowest concentration of amphotericin B, natamycin, or thimerosal that prevented any discernible growth.

The MIC range and mode, the MIC for 50% of the strains tested (MIC₅₀), and the MIC₉₀ were provided for the isolates with the SPSS statistical package (version 13.0). For calculation, any high off-scale MIC was converted to the next higher concentration.

The *in vitro* activities of thimerosal, amphotericin B, and natamycin against the isolates are summarized in Tables 1 and 2. When comparing the MIC₉₀s of thimerosal with those of natamycin and amphotericin B, the activity of thimerosal against *Fusarium* spp. is 256 times greater than that of nata-

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TABLE 1. *In vitro* susceptibilities of ocular *Fusarium* isolates to thimerosal, amphotericin B, and natamycin

Organism (no. of isolates) and antifungal agent	MIC range (mode) ^b	MIC ₅₀	MIC ₉₀
<i>Fusarium solani</i> species complex (82)			
Thimerosal	0.0078–0.0313 (0.0156)	0.0156	0.0313
Amphotericin B	0.5–16 (1)	1	2
Natamycin	4–32 (4)	4	8
<i>Fusarium moniliforme</i> species complex (20)			
Thimerosal	0.0156–0.0313 (0.0156)	0.0156	0.0313
Amphotericin B	1–8 (2)	2	2
Natamycin	4–8 (4)	4	8
<i>Fusarium avenaceum</i> species complex (16)			
Thimerosal	0.0156–0.0313 (0.0156)	0.0156	0.0313
Amphotericin B	0.5–8 (2)	2	4
Natamycin	4–32 (8)	8	8
Other <i>Fusarium</i> isolates (18) ^a			
Thimerosal	0.0078–0.0625 (0.0156)	0.0156	0.0313
Amphotericin B	0.5–2 (1)	1	2
Natamycin	4–8 (4)	4	8
<i>Fusarium</i> spp. (136)			
Thimerosal	0.0078–0.0625 (0.0156)	0.0156	0.0313
Amphotericin B	0.5–16 (1)	1	2
Natamycin	4–32 (4)	4	8

^a Includes 9 strains of *Fusarium oxysporum* species complex, 5 strains of *Fusarium poae* species complex, and 4 strains of *Fusarium lateritium* species complex.

^b Values are in micrograms per milliliter.

mycin and 64 times greater than that of amphotericin B, the activity of thimerosal against *Aspergillus* spp. is 512 times greater than that of natamycin and 32 times greater than that of amphotericin B, and the activity of thimerosal against *Alternaria alternata* is 128 times greater than that of natamycin and 32 times greater than that of amphotericin B. Therefore, thimerosal's effect was significantly superior to those of amphotericin B and natamycin against main ocular pathogenic fungi *in vitro*.

As shown in Tables 1 and 2, thimerosal has activity against different *Aspergillus* and *Fusarium* complexes. For each of these genera, this activity remains consistent and does not show significant interspecies variability. On the other hand, natamycin shows various activities against different *Aspergillus* spp. Most *Aspergillus* spp. are not susceptible, but *Aspergillus fumigatus* complex is susceptible to natamycin.

A noteworthy finding is that thimerosal exhibits the greatest activity against *Fusarium* spp. in comparison to the effects of all of the antifungals studied *in vitro* to date. Some studies (10–12) of the *in vitro* efficacy of traditional and newer antifungals against keratitis and endophthalmitis fungal pathogens show that amphotericin B and voriconazole have the lowest MIC₉₀s (2 to 4 µg/ml) against *Fusarium* spp., closely followed by terbinafine (8 µg/ml), natamycin (16 µg/ml), posaconazole (>8 µg/ml), itraconazole (>16 µg/ml), ketoconazole (>16 µg/ml), caspofungin (>16 µg/ml), 5-flucytosine (>64 µg/ml), and fluconazole (>256 µg/ml). When comparing the MIC₉₀s of thimerosal with those of other antifungals, the activity of thimerosal against *Fusarium* spp. is 64 to >8,179 times greater than those of other antifungals. It is very important because *Fusarium* spp. remain the most frequently isolated ocular fungal pathogens in China, Portugal, Singapore, Australia, and the

southern United States (2, 7, 12, 16, 18, 19, 23, 24, 26, 30) and the second most frequently isolated ocular fungal pathogens in India, Nepal, and Saudi Arabia (4, 8, 9, 17).

Successful treatment of otomycosis with thimerosal has been reported by Tisner et al. (21). Recently, our primary work based on clinical trials addressed the suitability of thimerosal for the treatment of keratitis. At the Henan Eye Institute and the Anyang Eye Hospital, 21 patients with filamentous keratomycosis were treated with thimerosal because they were not improving after topical amphotericin B, ketoconazole, and natamycin treatment for 1 to 3 weeks. Twenty of the 21 infections responded well to thimerosal. The keratomycosis healed after topical thimerosal treatment for 14 to 45 days (unpublished data).

Thimerosal is one of the main preservatives used worldwide in topical ophthalmic preparations, at concentrations ranging from 0.004 to 0.01%. Thimerosal has generally been accepted as a safe preservative agent in eye drops, and ocular side effects due to thimerosal are rare. No toxic effects have been observed following topical application of solutions containing thimerosal, even at concentrations 100 times higher than those required for a bactericidal effect (1, 5, 6). The findings from our study indicate that products formulated with thimerosal as both the main drug and a preservative can probably be used to treat keratomycosis successfully. We think that thimerosal has both antifungal and preservative effects without exceeding its value as a preservative and that the benefits of treating keratomycosis outweigh the potential risks for thimerosal.

In conclusion, in this study, thimerosal exhibited potent *in vitro* activity against main ocular pathogenic fungi and was even more effective than amphotericin B and natamycin. The

TABLE 2. *In vitro* susceptibilities of ocular *Aspergillus* and *Alternaria alternata* isolates to thimerosal, amphotericin B, and natamycin

Organism (no. of isolates) and antifungal agent	MIC range (mode) ^b	MIC ₅₀	MIC ₉₀
<i>Aspergillus flavus</i> species complex (49)			
Thimerosal	0.0313–0.0625 (0.0625)	0.0625	0.0625
Amphotericin B	1–32 (2)	2	2
Natamycin	8–32 (32)	32	32
<i>Aspergillus fumigatus</i> species complex (11)			
Thimerosal	0.0156–0.0625 (0.0313)	0.0313	0.0625
Amphotericin B	0.5–4 (1)	1	2
Natamycin	4–32 (4)	4	4
<i>Aspergillus oryzae</i> species complex (12)			
Thimerosal	0.0156–0.0625 (0.0625)	0.0625	0.0625
Amphotericin B	1–2 (1)	1	2
Natamycin	4–32 (32)	32	32
<i>Aspergillus versicolor</i> species complex (12)			
Thimerosal	0.0078–0.0625 (0.0078)	0.0156	0.0625
Amphotericin B	0.5–2 (1)	1	2
Natamycin	4–32 (8)	8	32
Other <i>Aspergillus</i> isolates (14) ^a			
Thimerosal	0.0078–0.0625 (0.0156)	0.0156	0.0313
Amphotericin B	0.125–2 (1)	1	2
Natamycin	0.25–32 (4)	4	32
<i>Aspergillus</i> spp. (98)			
Thimerosal	0.0078–0.0625 (0.0625)	0.0625	0.0625
Amphotericin B	0.125–32 (1)	1	2
Natamycin	0.25–32 (32)	32	32
<i>Alternaria alternata</i> (10)			
Thimerosal	0.0078–0.0625 (0.0156)	0.0156	0.0313
Amphotericin B	0.0625–2 (0.125)	0.125	1
Natamycin	2–8 (4)	4	4

^a Includes 8 strains of *Aspergillus niger* species complex, 2 strains of *Aspergillus candidus*, 2 strains of *Aspergillus nidulans*, 1 strain of *Aspergillus ochraceus*, and 1 strain of *Aspergillus wentii*.

^b Values are in micrograms per milliliter.

results suggest that thimerosal might play a role as a main drug in the treatment of keratomycosis and should be subjected to a prospective evaluation of efficacy and safety to further develop its clinical applications.

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