

***In vitro* Susceptibility of Fungal Isolates of Keratomycosis to Antiseptic Agents**

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Abstract

Background: Fungal corneal ulcer is the most dangerous and challenging type of infective keratitis. Since most of the ophthalmic antifungal drops are scarce and expensive in developing countries, attempts have been made to study fungicidal property of some readily available antiseptic agents as a substitute. Povidone iodine (PI) and chlorhexidine gluconate (CHx) has been postulated to be effective against fungi.

Objective: To study *in vitro* antifungal efficacy of PI and CHx.

Methods: Fungi isolated from cases of keratomycosis were entered in a prospective study from June 2001 to March 2002. *In vitro* susceptibility of these fungi was tested by broth dilution method of NCCLS Standard to PI (1%, 2%, 5%, 10%) and CHx (0.04%, 0.1%, 0.2%) after 5 minutes, 1 hr, 24 hrs and 48 hrs exposure times.

Results: From a total of 16 culture-proven cases of fungal keratitis, the isolated fungi were 8 *Aspergillus* sp, 3 *Fusarium* sp, 2 sterile hyphae, 1 *Candida* sp, 1 *Drechslera* sp, 1 *Rhodotorula* sp. PI showed 100% fungicidal effect with all tested concentrations, after 5 minutes of exposure to all fungal species.

CHx 0.1% and 0.2% after ≥ 1 hr exposure were as effective as PI ($p > 0.34$). The fungicidal efficacy of CHx 0.1% and 0.2% was significantly less than PI after 5 minutes ($p < 0.001$).

Conclusion: Both PI and CHx have strong *in vitro* fungicidal effect. The kill rate of CHx, however, is less than PI. Since *in vitro* efficacy of topical ophthalmic preparations is affected by multiple factors, our study provides a good idea for further *in vivo* investigations about this subject.

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Keywords • Fungal keratitis • keratomycosis • chlorhexidine • povidone iodine • fungal susceptibility test

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Introduction

Fungal keratitis constitutes 6-20% of infective keratitis. It is one of the most challenging forms of corneal ulcer both in regards to diagnosis and successful treatment³. Without early diagnosis and prompt treatment, it is not uncommon for the patient to lose vision or even the eye itself. Fungal keratitis (keratomycosis) is more prevalent in tropical areas. The diagnosis and

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treatment may pose difficult problem especially in the developing countries. Most of the topical antifungal medications are either not easily available or expensive and with variable efficacy. Three main groups of antifungal agents are approved for keratomycosis: 1) Polyenes (natamycin, amphotericin B) 2) Azoles (ketoconazole, fluconazole, itraconazole), and 3) Pyrimidines.¹

The antifungal efficacy of various antiseptic agents have been reported in previous reports.^{4,8} The results have been more promising for povidone iodine (PI) and chlorhexidine gluconate (CHx).^{2,3}

These agents have been approved for ophthalmic use. PI eye drop has been used as prophylactic medication to prevent postoperative endophthalmitis and neonatal conjunctivitis

CHx is used for the treatment of acanthamoeba keratitis.⁴ It is also used as a preservative in the eye drops and as a contact lens disinfectant.⁴

These agents are inexpensive and easily available. They could be a good substitute of the current antifungal drops if their antifungal efficacy is proved. We conducted a prospective study, to assess the *in vitro* fungicidal effect of PI and CHx on the fungal isolates from corneal ulcers.

Material and Methods

One hundred eyes of 99 patients who referred to Ophthalmology Emergency Service of Khalili Hospital from June 2001 to March 2002, with a clinical picture of infective corneal ulcer were enrolled in this study. A complete history and ophthalmic examinations including a drawing from the ulcer, the size (mm²), depth, color, shape and location was done. Then, under sterile condition and instillation of one drop of topical anesthetic, corneal scraping was performed for direct smear and culture. Scraping was performed by No 15 Bard-Parker blade from the edge and the depth of the ulcer. In some cases, corneal biopsy or penetrating keratoplasty was performed and the specimens were sent for culture and histopathologic diagnosis. The specimens were directly transferred into four culture medias comprising one blood agar, one chocolate agar and two cyclohexamide free Sabourad's dextrose agar (SDA). When specimen was scant, the primary isolation was performed in brain-heart infusion broth and then any growth was transferred into solid media. The plates were incubated at 37 °C and one of SDA plates at 25 °C. Direct smears were taken for Gram and Giemsa stains and KOH mount. The cultures were considered as positive for fungi, when more than two identical colonies were found on more than two plates.

Identification of the isolated fungi was done according to the growth rate, gross colony morphology, microscopic morphology and biochemical

characteristics. We performed subculture to enhance the development of the characteristic features for precise identification of the fungal species. Microscopic morphology of the fungi was identified by preparing a lactophenol cotton blue (LPCB) mount and suspending a tiny portion of fungal colony onto it. If adequate information was not obtained from LPCB mount, slide culture was performed for precise identification of the filamentous fungi.

The diagnosis of fungal keratitis was confirmed by observation of fungi either in KOH preparation, Giemsa stain, or histopathologic study of corneal biopsy or corneal button after penetrating keratoplasty (PKP) accompanied by typical or suspicious clinical picture.

Preparation of inoculum

The yeast isolates were transferred by sterile wire loop, from 18-48 hr-old cultures of SDA into a tube containing 2 ml of distilled water. The concentration was adjusted to about 1×10^6 cells/ml by spectrophotometer. The spectrophotometer was set at 530 nm and the suspension was adjusted on 90% transmittance, which is compatible with the desired dilution of the suspension. To prepare the suspension of the filamentous fungi, the inoculum was obtained from 2-5 day-old cultures on SDA. The fungal suspension was prepared by immersing the surface growth into sterile distilled water containing 0.05% Tween 80. The spores and hyphae were transferred by sterile wire loop into the test tubes and the suspension diluted up to 1×10^6 colony forming units (CFU)/ml by 90% transmittance of spectrophotometer set at 530 nm.

Preparation of the drug solutions

Two kinds of PI were used: 1) Betadine™ made by Seton Health Care Group PIC Tubition House, Oldham, UK, and 2) PI 10% made by Tolid Daru Co. Tehran, Iran.

The PI solutions were diluted to 1%, 2% and 5% concentrations with sterile distilled water. CHx 0.2% made by Daru Shahr, Thran, Iran was diluted to 0.1% and 0.04% concentrations.

Test Method

For each fungal isolate, 14 capped test tubes (110×16 mm) were prepared; four for the UK-made PI and four for the Iranian PI. Each tube contained one of 0.1%, 0.2%, 5% and 10% concentrations. Three tubes contained either 0.04%, 0.1% or 0.2% concentrations of CHx.

One tube serving as positive (+ve) control and contained fungi in distilled water without any agent. Two tubes served as negative (-ve) control; one contained PI without fungi and another, CHx without fungi.

One-tenth ml of the agent to be tested was added to the related tube except for the +ve control tube. Test tubes were incubated at 37 °C and were examined four times; on 5 minutes, 1 hr, 24 hrs, and 48 hrs after incubation. Each time, a sample was taken from the tube and added to a SDA plate. The plate was incubated at 30 °C for 10 days and observed every day to monitor any growth of fungal elements. To prove the accuracy of the procedure, it was expected to observe growth of fungus in the cultures of the samples taken from the +ve control tube and no growth from the -ve control tubes.

Results

A total of 16 positive fungal cultures were obtained from 100 eyes of 99 patients with infective corneal ulcer (16%). The isolated fungi comprised 7 *Aspergillus niger*, 1 *Aspergillus falvus*, 3 *Fusarium* spp., 2 sterile hyphae, 1 *Candida* spp., 1 *Rhodotorula* spp. and 1 *Drechslera* spp.

The results of the fungal susceptibility test were the same for both kinds of PI (UK and Iranian). All of the inoculated fungi were killed at all concentrations (1%, 2%, 5%, and 10%) of this agent as early as 5 minutes after exposure.

All of the +ve control tubes showed +ve fungal culture and all of the -ve control tubes were culture -ve. The fungicidal efficacy of CHx was dose and time dependent. Its efficacy was not acceptable for 0.04% concentration. There was no statistically significant difference between 0.1% and 0.2% concentrations. Their effect was not acceptable after 5 minutes, but satisfactory after 1 hr. The results are summarized in Table 1.

Discussion

The results of this study are indicative of high percentage of fungal keratitis in this sample of 100 cases of corneal ulcer from southern states of Iran. The percentage is comparable to some reports from India,¹² where the largest series of this infection has been reported.¹³

The most common cause of fungal keratitis throughout the world, is *Aspergillus* spp. *Fusarium* spp. is more prevalent in warm climates and Can-

ada in cool areas like northern US and Canada.^{1,3}

In our study, *Aspergillus* and *Fusarium* were the most frequent isolates. Two unusual species (*Rhodotorula* and *Drechslera*) were also isolated. Fungal isolates from two cases of fulminant corneal ulcer were sterile septated hyphae. They could not be identified due to the lack of spore and conidia formation despite several subcultures and trials.

Regarding the method for antifungal susceptibility test, it should be mentioned that these tests are not routinely performed because of technical difficulties. Three main techniques of fungal sensitivity test have been performed in earlier studies; 1) Broth dilution method, 2) E-test, and 3) Agar diffusion method. Broth dilution is the most accurate and standardized technique.⁹⁻¹¹ This method could determine the minimum fungicidal concentration (MFC). The limitation of this method is that, it is time consuming and technically difficult.

The E-Test method is performed by standard strips (made by AB, B10 Disc, Solona, Sweden). This method is easy and accurate. However, the strips are made only for five specific antifungal drugs and are not available for PI and CHx.¹⁴

The agar diffusion method⁴ is performed by filling the wells into the agar culture plates, by the agents to be tested. This technique is simple and cheap. Disadvantage of this method, however, is that penetration and diffusion of the agent in the agar affect the concentration of the drug in the agar around the wells and is variable for different agents. This problem may create significant errors.

The results of fungal susceptibility test in our study showed that PI possesses a very strong and rapid fungicidal activity while the antifungal efficacy of CHx was dose and time related.

The fungicidal activity of CHx was promising only at 0.1% and 0.2% concentrations, but not acceptable for 0.04% concentration. The kill rate of CHx was less than PI. The fungicidal effect of 0.1% and 0.2% CHx was significantly low after 5 minutes of exposure to fungi, but acceptable after one hour. The point that should be mentioned is that the eye drops exit the conjunctival space within 5-10 minutes after instillation via the nasolacrimal duct. So, the *in vivo* efficacy of topical ophthalmic medications may be affected by their kill rate. Some other factors are also influential over the exposure time of the organism to the topical medication. These are the penetration rate of drug into the cornea and frequency of the drug instillation.

In vitro antifungal efficacy of CHx and PI has been studied in a report from Ghana and India⁴ In this study, the fungicidal efficacy of CHx was not acceptable for the concentrations under 0.2%. PI 2.5% was as effective as CHx 0.2% with 95% efficacy. Their test method was agar diffusion which is

Table 1: Antifungal efficacy (%) of chlorhexidine gluconate at different concentrations

Concentration (%)	Exposure Time			
	5 min	1 hr	24 hr	48 hr
0.04	19	38	50	50
0.1	25	88	100	100
0.2	38	88	100	100

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not very precise. This report was followed by a clinical pilot study on 15 patients with fungal keratitis. Surprisingly, PI failed to treat the fungal corneal ulcers while the CHx was effective.⁵

In another clinical trial, in 5 out of 44 culture-proven cases of fungal keratitis, the efficacy of CHx was equal to natamycin (the most popular specific anti-fungal eye drop).

These studies indicate that the clinical outcome may not always parallel the *in vitro* sensitivity, however the results of *in vitro* study provides a primary idea as to the potential fungicidal efficacy of antiseptic agents and spectrum of their effect on various fungal species. This is a starting point for designing animal experiments and further clinical trials to investigate the *in vivo* efficacy of these agents. If the *in vivo* studies prove the fungicidal efficacy and practical usefulness of these agents, they would be very appropriate substitutes for the present topical antifungal ophthalmic drugs.

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