

Antifungal susceptibility testing of yeast isolated from corneal infections

Teste de susceptibilidade a antifúngicos de leveduras isoladas de infecções corneais

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ABSTRACT

Purpose: To report the antifungal susceptibility profile of yeast isolates obtained from cases of keratitis. **Methods:** Susceptibility testing of 15 yeast strains isolated from corneal infections to amphotericin B, fluconazole, itraconazole and ketoconazole was performed using the NCCLS broth microdilution assay. **Results:** Most episodes of eye infections were caused by *Candida albicans*. The antifungal drugs tested showed the following minimal inhibitory concentration values against yeast isolates: 0.125-0.5 µg/ml for amphotericin B; 0.125->64.0 µg/ml for fluconazole; 0.015-1.0 µg/ml for itraconazole and 0.015-0.125 µg/ml for ketoconazole. Despite the fact that all *Candida* isolates were judged to be susceptible to azoles, one isolate showed a minimal inhibitory concentration value significantly higher than a 90% minimal inhibitory concentration of all tested isolates. *Rhodotorula rubra* was resistant to fluconazole and itraconazole. **Conclusions:** Despite the fact that most yeast isolates from corneal infections are usually susceptible to amphotericin B and azoles, they exhibit a wide range of minimal inhibitory concentration values for antifungal drugs. The identification of strains at species level and their susceptibility pattern to antifungal drugs should be considered before determining the concentration to be used in topical antifungal formulations in order to optimize therapeutic response in eye infections.

Keywords: Eye infections, Keratitis, Antifungal agents, Yeasts infections, Antifungal susceptibility tests.

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INTRODUCTION

Fungi are opportunistic agents which rarely infect healthy corneas of immunocompetent individuals. Systemic and local underlying diseases may render the corneal tissues susceptible to infections with these organisms⁽¹⁾. Trauma, specially related to organic materials, topical use of steroids and antibiotics⁽²⁻³⁾, contact lens wear, surgical procedures, contamination of eye solutions, inappropriate maintenance of contact lenses⁽⁴⁾, systemic infections as well as any disease associated with immunodeficiency are considered risk factors for fungal eye infections⁽⁵⁾.

In Brazil, the incidence of fungal eye infections ranges from 3.8 to 10%, depending on the site of the infection, the diagnostic tools available for fungal infections in the medical center and the seasons related to the studied period⁽⁶⁻⁹⁾.

The occurrence of keratomycosis is of great importance, not only because of diagnostic problems but also due to the severity of clinical manifestations and poor outcome despite antifungal treatment. The severity and progression of the fungal infection usually depend on the existence of

previous underlying diseases, impaired local defense mechanisms and the involved fungal species⁽¹⁰⁻¹¹⁾.

Therapeutic failure rates in fungal keratitis are very high in different series and the poor outcome is related to several factors including: late diagnosis, host's underlying diseases, reduced ocular drug penetration and low antifungal susceptibility of certain etiologic agents^(2,10,12-15).

Despite the fact that fungal eye infections continue to be a challenge to ophthalmologists due to the high rates of therapeutic failures⁽¹⁶⁾ observed in different services, we found few studies referring to *in vitro* antifungal susceptibility testing of clinical fungal isolates obtained from episodes of eye infections⁽¹⁶⁾.

The purpose of this study was to evaluate the antifungal susceptibility profile of yeast isolates from corneal infections using the internationally accepted NCCLS methodology.

METHODS

From 1996 to 1998, corneal yeast infections were diagnosed in 15 patients attended at the External Eye Disease and Corneal Services of the Department of Ophthalmology, of the Federal University of São Paulo. Isolation of the involved etiologic agent as well as the stocking of the specimens were performed at the Laboratory of Microbiology of this department. The patients' main demographic and clinical data were obtained by reviewing their charts.

All yeast isolates were submitted to species identification and *in vitro* antifungal susceptibility tests at the Special Mycology Laboratory of the Division of Infectious Diseases of the Department of Medicine.

Identification of the yeast was based on their micromorphology on Cornmeal-Tween 80 agar and their biochemical profile was determined by assimilation and carbohydrate fermentation tests⁽¹⁷⁾.

Antifungal susceptibility tests were performed using the broth microdilution assay according to the methodology recommended by the NCCLS (1997)⁽¹⁸⁾. The following antifungal drugs, supplied by the manufacturers as pure standard compounds, were tested: amphotericin B, fluconazole, itraconazole and ketoconazole. Briefly, the medium used was RPMI-1640, with L-glutamine, without bicarbonate, and buffered at pH 7.0 with 0.165 M MOPS. The yeast inoculum was prepared based on optical density using a spectrophotometer to obtain a yeast concentration of 0.5 - 2.5 x 10³ cells/ml. The assays were incubated at 35°C for 48 h. The minimal inhibitory concentration (MIC) for amphotericin B was considered the lowest tested concentration able to inhibit any visual colony growth. MIC for azoles was considered the lowest concentration able to inhibit 50% colony growth relative to control.

The breakpoints used for the definition of susceptibility categories are those proposed by the NCCLS and are as follows:

Fluconazole: susceptible if MIC < 8.0 µg/ml, dose dependent susceptibility (DD-S) if MIC from 16.0 to 32.0 µg/ml and resistant if MIC > 64.0 µg/ml;

Itraconazole: Susceptible if MIC < 0.125 µg/ml, dose-dependent susceptibility if MIC from 0.25 to 0.50 µg/ml and resistant if MIC > 1.0 µg/ml.

Due to a lack of consensus about the definition of MIC breakpoint for ketoconazole and amphotericin B, arbitrary values were established according to suggested values of previous studies:

Ketoconazole: susceptible for isolates with < 0.125 µg/ml, DD-S for isolates with MIC between 0.25 and 0.50 µg/ml and resistant for isolates with MIC > 1.0 µg/ml;

Amphotericin B: susceptible if MIC < 1.0 µg/ml and resistant if MIC > 2.0 µg/ml.

RESULTS

Clinical and epidemiological characteristics of the evaluated patients are summarized in Table 1.

The patients' ages ranged from 10 to 89 years, with a median age of 64 years, $\bar{x} = 56.4$) with 6 episodes (40%) occurring in males. It was possible to identify one or more predisposing factors for eye infection in all enrolled patients, with an emphasis on local immunosuppression as well as the use of topical antibiotics.

The species distribution of the yeast isolates, as shown in Table 1, was the following: *C. albicans* (53.3%), *C. parapsilosis* (20.0%), *C. tropicalis* (20.0%) and one case of *Rhodotorula rubra*.

The MIC values of antifungal drugs tested are shown in Table 2.

The MIC values of the antifungal drugs tested against *Candida* spp isolates ranged from 0.125 to 0.5 µg/ml for amphotericin B, 0.125 to 4.0 µg/ml for fluconazole, 0.015 to 0.25 µg/ml for itraconazole and 0.015 µg/ml to 0.06 µg/ml for ketoconazole.

Using the susceptibility breakpoints suggested by the NCCLS⁽²¹⁾ for the studied antifungal drugs, it was possible to state that all samples were susceptible to amphotericin B. Regarding azoles, the *Rhodotorula rubra* isolate was resistant to fluconazole and itraconazole. Similarly, one *C. albicans* strain exhibited dose-dependent susceptibility to itraconazole. It should be mentioned that although all *Candida* isolates were susceptible to fluconazole, one *C. albicans* isolate showed a MIC value of 2.0 µg/ml (patient 3) for fluconazole and one *C. parapsilosis* strain exhibited a MIC value of 4.0 µg/ml for fluconazole (patient 10).

Table 3 summarizes the MIC ranges, MIC-50% (concentration able to inhibit 50% colony growth (or colony formation) of the tested samples) and MIC-90% (concentration able to inhibit 90% colony growth (or colony formation) of the tested samples) values of antifungal drugs tested against all *C. albicans* isolates.

Table 1. Clinical, epidemiological and laboratory data of 15 episodes of corneal infections attended at the Department of Ophthalmology, UNIFESP – EPM (1996 to 1998)

No.	Age (years)	Sex	Predisposing factors	Site	Microorganism
1	74	F	Corneal surgery - Tx	Cornea	<i>C. albicans</i>
2	45	F	Trauma – Foreign body	Cornea	<i>C. albicans</i>
3	81	M	Exposed ulcer	Cornea	<i>C. albicans</i>
4	89	F	Senility	Cornea	<i>C. albicans</i>
5	69	M	Corneal perforation	Cornea	<i>C. albicans</i>
6	47	M	Endophthalmitis/keratitis	Cornea	<i>C. albicans</i>
7	44	F	Immunosuppression Sjögren S.	Cornea	<i>C. albicans</i>
8	44	M	Immunosuppression HIV +	Cornea	<i>C. albicans</i>
9	70	F	Corneal surgery - ulcer	Cornea	<i>C. parapsilosis</i>
10	83	F	Use of topical medication	Cornea	<i>C. parapsilosis</i>
11	48	F	Corneal surgery – Tx	Cornea	<i>C. parapsilosis</i>
12	14	M	Trauma	Cornea	<i>C. tropicalis</i>
13	64	F	Surgery - Tx	Cornea	<i>C. tropicalis</i>
14	64	F	Surgery - Tx	Cornea	<i>C. tropicalis</i>
15	10	M	Dermatological alteration	Cornea	<i>Rhodotorula rubra</i>

F - Female; M - Male; Tx - Corneal transplantation; C. - *Candida*

Table 2. Minimal inhibitory concentration (MIC) values of 4 antifungal drugs tested on 15 yeast specimens

No.	MIC values (µg/ml)				Microorganisms
	Amphotericin-B	Fluconazole	Itraconazole	Ketoconazole	
1	0.25	0.5	0.03	0.015	<i>C. albicans</i>
2	0.25	2.0	0.25 (I)	0.06	<i>C. albicans</i>
3	0.125	0.25	0.015	0.015	<i>C. albicans</i>
4	0.5	0.25	0.03	0.03	<i>C. albicans</i>
5	0.125	0.125	0.06	0.015	<i>C. albicans</i>
6	0.5	0.125	0.015	0.015	<i>C. albicans</i>
7	0.25	0.125	0.015	0.015	<i>C. albicans</i>
8	0.5	0.25	0.03	0.03	<i>C. albicans</i>
9	0.25	0.5	0.06	0.015	<i>C. parapsilosis</i>
10	0.125	4.0	0.06	0.06	<i>C. parapsilosis</i>
11	0.5	0.5	0.03	0.015	<i>C. parapsilosis</i>
12	0.25	0.25	0.015	0.015	<i>C. tropicalis</i>
13	0.125	1.0	0.06	0.03	<i>C. tropicalis</i>
14	0.25	0.125	0.015	0.015	<i>C. tropicalis</i>
15	0.25	>64 ®	1.0 ®	0.125	<i>R. rubra</i>

MIC – Minimal inhibitory concentration; C. – *Candida*; R. *Rhodotorula*; ® – Resistant; (I) – Dose dependent susceptibility

DISCUSSION

Although mycotic keratitis may occur in a variety of climatic zones, it seems to be more common in regions with subtropical or tropical climate, both found in the greater part of Brazil. Fungi accounted for 5% of all infectious keratitis (61 of 1113 patients) as documented in the Laboratory of External Diseases of the Department of Ophthalmology, Federal University of São Paulo - Escola Paulista de Medicina, from 1995 to 1998 (data not shown). *Fusarium* genus (51%), *Candida* spp (23%) and *Aspergillus* spp (8.19%) were the most commonly isolated agents of fungal keratitis⁽¹⁹⁾.

In the present study, the clinical and microbiological characteristics of documented yeast keratitis were evaluated at

the institution. In agreement with other series, all patients with yeast corneal infections presented with at least one predisposing factor to corneal fungal infections, including the use of antibiotics and corticoids, systemic immunosuppression, advanced age, trauma or previous surgery.

Regarding the etiology of the eye infections, 53.3% was keratitis caused by *C. albicans*. This finding is in agreement with previous publications addressing the etiology of eye infections. Rosa et al.⁽¹⁹⁾ reviewed the charts of 125 patients with fungal keratitis in South Florida for a period of 10 years (1982-1992) and observed that the *Candida* genus accounted for 12% (16 cases) of all infections. Regarding species, *C. albicans* corresponded to 25%⁽²⁰⁾. Wakisaka et al, after analyzing the cultures of corneal ulcers in 263 patients, from

September, 1986 to February, 1998, found 10 episodes of mycotic keratitis (4%) where 2 cases were caused by *C. albicans* isolates⁽⁶⁾. In this laboratory, a retrospective study was conducted to evaluate 671 cases of infectious corneal ulcers reported during a period of 10 years (January, 1981 to December, 1991). The authors found that 64 episodes (1.5%) were caused by fungi and *Candida sp* accounted for 12.5% of all identified fungal isolates⁽⁹⁾.

It is interesting that we were able to identify one case of keratitis due to *Rhodotorula rubra*, an agent of low pathogenicity and virulence, causing corneal infection. We did not find any previous report of keratitis caused by this yeast pathogen. However, different authors view this agent as being an emergent pathogen among superficial and systemic fungal infections⁽²¹⁻²³⁾.

To enhance drug penetration and clinical efficacy, keratitis therapy involves the use of topic, systemic and subconjunctival medication after removal of the corneal epithelium. Topical amphotericin B is considered to be the drug of choice for the treatment of mycotic keratitis⁽²⁴⁾. The range of amphotericin B concentrations in topic formulations used for the treatment of corneal infections is very broad. Experiments have shown that concentrations between 0.015 and 1% may be used. Amphotericin B is toxic when administered by the subconjunctival route and severe complications, including conjunctival necrosis, may occur⁽¹⁴⁾. Intravenous use of amphotericin B is limited in the case of fungal keratitis because, besides all the systemic adverse effects, it penetrates the cornea minimally reaching low levels in the aqueous humor⁽¹⁵⁾. After evaluating the susceptibility profile of amphotericin B obtained with the tested clinical yeast isolates, we found that all were susceptible to this drug; however, a MIC variation of 0.125 µg/ml to 0.5 µg/ml was observed. Considering the range of MIC obtained with amphotericin B, to optimize the clinical efficacy of topic formulations and avoid toxicity, it would be reasonable to propose that patients infected with agents with higher susceptibility to this drug should be treated with lower concentrations and vice-versa. This notion needs to be validated by clinical and experimental studies which are only possible with the use of susceptibility tests applied to ocular pathogens.

Ketoconazole is an imidazole to be used orally, truly requiring systemic absorption which depends on the gastric acidity. Topical formulations of ketoconazole may be prepared at a 1 to 5% concentration, reaching good therapeutic corneal levels. Since ketoconazole can be administered orally and shows a wide antifungal spectrum, the drug is a natural candidate for use in the systemic treatment of keratomycosis, either

alone or in association with topic preparations⁽¹⁵⁾. In the present study, the isolates were all susceptible to ketoconazole but exhibited MIC values ranging from 0.015 to 0.125 µg/ml, a variation of almost 100 times the inhibitory potential of this drug in relation to the examined pathogens.

Fluconazole is a triazole exhibiting excellent tolerability and intraocular penetration. However, its antifungal activity is basically restricted to the genus *Candida*, *C. neoformans* isolates and dermatophytes. It is important to note that fluconazole has no antimycotic activity against *Aspergillus* spp and *Fusarium* spp, the agents which usually cause keratitis^(15,25). Regarding fluconazole MIC, we obtained a large variation of values against the tested ocular pathogens, including one *C. albicans* strain that exhibited a MIC value higher than the MIC-90 for the 8 tested samples (Table 3). On the other hand, there was one episode of *R. rubra* keratitis in which the isolate exhibited *in vitro* resistance to fluconazole and itraconazole. These data reinforce the potential role of susceptibility tests in defining a rational therapeutic strategy for fungal keratitis.

Itraconazole is a triazole with a large spectrum of antifungal activity including activity against the genus *Candida*, *C. neoformans* isolates, as well as dermatophytes, *Aspergillus* spp and dimorphic fungi. However, the capsule formulation available shows bioavailability limitations affecting its serum levels and tissular distribution. Therefore, in spite of its high tolerability and broad spectrum, its clinical ophthalmologic use is restricted⁽²⁵⁻²⁶⁾. In the present study, MIC values for itraconazole were quite lower than those recorded for fluconazole when we evaluated *Candida* spp specimens. These data reinforce the therapeutic potential of itraconazole in fungal keratitis, whose clinical applicability could be broadened with the recent advent of oral and parenteral solutions of this derivative solubilized in cyclodextrin⁽²⁷⁾.

Recently, several studies have called attention to the occurrence of primary or acquired infections due to yeast resistant to antifungal drugs. Such reports are more frequent among patients with AIDS or malignant hematological diseases, situations in which the prophylactic or therapeutic use of antimycotics is more disseminated⁽²⁸⁻³⁰⁾. The literature includes many studies which evaluate the susceptibility profile of yeast isolates from different biological fluids and tissues; however, none were found describing the antifungal susceptibility profile of yeast isolates from ocular infections⁽³¹⁻³⁵⁾.

Considering the high therapeutic failure rate associated with ocular fungal infections, the investigation of the antifun-

Table 3. MIC ranges, MIC 50% and MIC 90% values of 4 antifungal drugs against 8 *C. Albicans* isolates

Variables/Antifungals	Amphotericin B	Fluconazole	Itraconazole	Ketoconazole
MIC ranges (µg/ml)	0.125 - 0.5	0.125 - 2.0	0.015 - 0.25	0.015 - 0.06
MIC - 50%	0.25	0.25	0.03	0.015
MIC - 90%	0.50	0.50	0.06	0.03
MIC - Minimal inhibitory concentration				

gal susceptibility profile of ocular fungal pathogens may be useful to identify antifungal resistance as a possible risk factor for unfavorable outcome. Similarly, to optimize the toxicity/therapeutic efficacy ratio with respect to ocular infections, it is reasonable to suggest that MIC values of antifungal drugs should be considered before defining the best concentration of the drug to be used in topic formulations. The actual benefit of such management should be evaluated by further clinical studies.

CONCLUSIONS

In conclusion, in spite of the fact that most yeast isolates from ocular infections were found to be susceptible to amphotericin B and azoles, a wide variation of antifungal MIC values were obtained with the various tested pathogens. These data provide a warning to reference laboratories associated with Ophthalmology Services that they should be prepared not only for the accurate identification of the fungi found in biological fluids, but also for determination of their susceptibility to antifungal drugs as an additional tool to support therapy.

RESUMO

Objetivo: Relatar resultados e avaliar a aplicabilidade do teste de suscetibilidade a antifúngicos de leveduras isoladas de infecções corneais oculares. **Métodos:** Realizou-se teste de suscetibilidade pelo método de microdiluição em caldo, padronizado pelo NCCLS-EUA, em 15 amostras de leveduras de infecções corneanas a anfotericina B, fluconazol, itraconazol e ketoconazol. **Resultados:** A maioria dos episódios de infecção corneal foi causada por *Candida albicans*. As drogas antifúngicas testadas exibiram valores de concentração inibitória mínima (CIM) para as leveduras isoladas: 0,125-0,5 µg/ml para anfotericina B; 0,125->64,0 µg/ml para fluconazol; 0,015-1,0 µg/ml para itraconazol e 0,015-0,125 µg/ml para ketoconazol. Apesar de todas as amostras terem sido suscetíveis aos azólicos, uma exibiu valor de concentração inibitória mínima significativamente maior do que a concentração inibitória mínima 90% de todas as amostras testadas. *Rhodotorula rubra* foi resistente a fluconazol e itraconazol. **Conclusão:** Apesar da maioria das leveduras isoladas de infecções corneais serem usualmente suscetíveis a anfotericina B e azólicos, observa-se ampla variação de valores de concentração inibitória mínima obtidas com as diferentes drogas antifúngicas testadas. A identificação das cepas e a determinação do padrão de suscetibilidade devem ser consideradas antes da determinação da concentração das drogas a ser empregada em formulações antifúngicas tópicas com o objetivo de otimizar a resposta terapêutica às infecções oculares.

Descritores: Infecções oculares; Ceratites; Antifúngicos; Infecções por leveduras; Testes de suscetibilidade a antifúngicos.

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