ORIGINAL ARTICLE

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EVALUATION OF THE DISK DIFFUSION METHOD FOR TESTING FLUCONAZOLE SUSCEPTIBILITY OF *Cryptococcus laurentii*

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ABSTRACT

Cryptococcus laurentii is a rare human pathogen ubiquitous in nature. This study aims to evaluate the disk diffusion method for testing fluconazole susceptibility of *C. laurentii*, and moreover, to assess the minimal fungicidal concentration (MFC) by the broth microdilution method. Eleven isolates of environmental *C. laurentii* complex were employed to determine the susceptibility to fluconazole by disk diffusion and by broth microdilution methods according to CLSI (Clinical and Laboratory Standards Institute) guidelines and to determine the MFC by broth microdilution technique. The disk diffusion method indicated four susceptible, three susceptible-dose dependent and four resistant isolates while by the broth microdilution method 10 isolates were defined as susceptible and one susceptible-dose dependent. The agreement between the methods was 36.4%. One isolate showed MFC of 8 µg/mL and two of 64 µg/mL. Although only a small number of isolates were studied, results suggested that the disk diffusion method was not adequate to determine *in vitro* susceptibility to fluconazole for *C. laurentii* isolates, and that fluconazole, while it is a fungistatic antifungal, may present *in vitro* fungicidal activity for some isolates.

KEY WORDS: *Cryptococcus laurentii*; antifungal testing; disk diffusion; fluconazole; minimal fungicidal concentration.

RESUMO

Avaliação do método de difusão do disco para o teste de sensibilidade ao fluconazol de isolados de *Cryptococcus laurentii*

Cryptococcus laurentii é um patógeno humano raro, ubíquo na natureza. O objetivo deste estudo foi avaliar a aplicação do método de difusão do disco para determinar a sensibilidade ao fluconazol

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de isolados de *C. laurentii* e determinar a concentração fungicida mínima (CFM) do fluconazol. Foi determinada a sensibilidade ao fluconazol pelos métodos de difusão do disco e microdiluição em caldo de 11 isolados de *C. laurentii*, de acordo com CLSI (Clinical and Laboratory Standards Institute), e a CFM pelo método de microdiluição em caldo. O método de difusão do disco mostrou quatro isolados sensíveis, três sensíveis dose-dependentes e quatro resistentes, enquanto que pelo método de microdiluição, 10 isolados foram sensíveis e um sensível dose-dependente. A concordância entre os dois métodos foi de 36,4%. Um isolado apresentou CFM de 8 μg/mL e dois de 64 μg/mL. Embora o número de isolados estudados seja pequeno, os resultados sugerem que o método de difusão do disco não deve ser usado na determinação da sensibilidade *in vitro* dos isolados de *C. laurentii* ao fluconazol, e apesar de ser uma droga fungistática, o fluconazol pode apresentar atividade fungicida *in vitro* para alguns isolados do complexo *C. laurentii*.

DESCRITORES: *Cryptococcus laurentii*; testes de sensibilidade; difusão do disco; fluconazol; concentração fungicida mínima.

INTRODUCTION

Encapsulated yeasts of the *Cryptococcus* genus are encountered worldwide in diverse ecosystems, and in both animals and avian excreta (9, 17, 21). *C. laurentii*, considered a rare human pathogen, is described as an infective agent in skin, in keratitis, oropharyngeal airways, endophthalmitis, pulmonary abscess, pneumonia, peritonitis, meningitis and fungaemia (2, 10, 11, 13, 14, 22, 25). In contrast to the *C. neoformans* complex, *C. laurentii* does not seem to have tropism for the central nervous system (16).

C. laurentii is a complex of species, as shown by DNA composition studies and electrophoresis patterns of cell proteins. Based on sequence analysis of the D1/D2 region of 26S rDNA and of regions of the internal transcribed spacer isolates of *C. laurentii* were divided into two phylogenetic groups, I and II (23, 24).

Patients infected with *C. laurentii* are treated with amphotericin B and fluconazole, using similar methodology to the treatment of infection with the *C. neoformans* complex, depending on the clinical conditions and the organ involved. *In vitro* cases of resistance to both antimicrobials are described but not frequent (12, 16).

The *C. laurentii* complex is not well studied, but the increasing number of infections it causes worldwide emphasizes the importance of efforts to provide better and rapid identification of isolates in clinical diagnosis laboratories. Standard criteria to determine *in vitro* susceptibility of *Cryptococcus* species are not established, but several methods such as broth microdilution, E-test®, Sensititre® and ATBFungus® are used (3, 8, 16).

The application of a simple, low cost method requiring only basic equipment would attract mycology laboratories to the task of determining the *in vitro* susceptibility of different fungi to antimicrobials. Thus, the purpose of the present study was to determine the *in vitro* susceptibility of *C. laurentii* isolates to fluconazole by the disk diffusion method, and to compare to the broth microdilution method. Additionally, we evaluated the fungicidal activity of fluconazole for *C. laurentii* isolates.

MATERIALS AND METHODS

Eleven environmental isolates of *C. laurentii* complex were included in this study. They were previously identified by classical methodology (capsule, phenoloxidase production on DOPA medium, urea hydrolysis, glucose fermentation, assimilation of carbon and nitrogen sources) and the API 20 *Candida* system (BioMerieux, Paris, France) (18, 19).

Susceptibility determinations by the disk diffusion method were conducted according to document M44-A2, from CLSI guidelines 2009 (7), with some modifications. Briefly, the yeast cell suspension (10^6 cells/mL) was spread with a cotton swab on the surface of a modified agar Müeller-Hinton plate (supplemented with 2% glucose and 0.5 µg/mL methylene blue). Fluconazole containing disks (25 µg) (Cecon, São Paulo, SP, Brazil) were applied on the plates, which were incubated at 30-32°C (8, 18). After 24 hours, inhibition of growth was assessed and if not adequate, incubation was extended to 48 hours. Cut off points utilized to classify diameter of inhibition halos were susceptible (S) when ≥ 19 mm, susceptible-dose dependent (S-DD) between 15-18 mm, and resistant (R) ≤ 14 mm (7). Standard strains, *Candida parapsilosis* ATCC 22019 and *Candida albicans* ATCC 90028, were controls.

Susceptibility determinations by the broth microdilution procedure were conducted according to document M27-A3, from CLSI guidelines 2008 (6), with modifications. Briefly, medium RPMI-1640 containing glutamine, free of sodium bicarbonate, buffered by MOPS, pH 7, was supplemented with 18 g/L glucose and the inoculum suspension adjusted to 1-5 x 10^6 cells/mL. The control was *C. parapsilosis* ATCC 22019. Inoculated plates containing the antimicrobial were incubated for 48 hours at 30°C (3, 8, 18). Minimal Inhibitory Concentration (MIC) was defined as the concentration of antifungal able to inhibit at least 50% of fungus growth (turbidity) in relation to the control. Fluconazole inhibition was graded as follows: susceptible (S) for a MIC ≤ 8 µg/mL, susceptible dose-dependent (S-DD) for MIC between 16-32 µg/mL, and resistant (R) for MIC ≥ 64 µg/mL

Categorical agreement (CA) was defined as the percentage of isolates classified in the same category by both methods. Discrepancies between methods were considered very major errors (VME) if an isolate classified as showing resistance *in vitro* by the MIC technique was categorized as susceptible by the other. Discrepancies were considered major errors (ME) if an isolate classified as susceptible by the MIC method was classified as resistant by the disk diffusion technique. Minor errors (MiE) were considered to have occurred when a susceptible isolate was classified as susceptible-dose dependent, when a resistant organism was grouped with S-DD isolates, or when S-DD isolates were classified as resistant organisms.

Minimal fungicide concentration (MFC) was determined by seeding 10 µL of the homogenized suspension from each well of the microdilution plate into

Sabouraud dextrose agar plates with increasing antimicrobial concentrations up to the last well. Plates were incubated at 30°C for five days. MFC corresponded to the well which did not contain viable cells, shown by the absence of growth of yeast colonies on the plates. All tests were made in duplicate.

RESULTS

Results of disk diffusion tests were shown after 24 hours by five isolates and after 48 hours by the remaining six because of the trailing effect and absence of growth for reading some isolates at 24 hours. Inhibition zone diameters for nine isolates varied from 12 mm and 40 mm (Table 1). MIC values ranged between 1 to 32 μg/mL, and MIC_{50} and MIC_{90} were both 8 μg/mL. Seven isolates with MIC of 8 μg/mL showed inhibition zone diameters between 12 mm to 22 mm, and one did not. No inhibition zone was detected in another isolate, which had a MIC of 32 μg/mL. Comparing the methods of disk diffusion and broth microdilution, the first indicated four susceptible isolates, three susceptible-dose dependent and four resistant, whilst 10 isolates were shown to be susceptible and one was dose-dependent by the second method. Regarding the categorical agreement (CA), only four isolates (36.4%) were susceptible by both methods demonstrating a concordance (CA) of 100%; percentages of CA were 27.3% and 36.4% for ME and MiE, respectively. VME were not observed.

The MFC was determined for three isolates; one had a MFC of 8 μg/mL and the other two of 64 μg/mL. For the remaining isolates fluconazole concentrations used in the study were not fungicidal (Table 1).

Table 1. Results of susceptibility tests to fluconazole by the methods of disk diffusion (DD) and broth microdilution (MIC), and minimal fungicide concentration (MFC) for 11 isolates of *Cryptococcus laurentii* complex.

	DD (mm)		MIC (μg/mL)	MFC (μg/mL)
	24 h	48 h		
01	15	-	8	> 64
02	12	-	8	> 64
04	-	22	8	64
05	17	-	8	64
08	-	40	1	8
09	-	18	8	> 64
10	-	00	32	> 64
11	-	00	8	> 64
12	21	-	8	> 64
13	13	-	4	> 64
15	-	22	4	> 64
C. albicans ATCC 90028	(-)	(-)	32	(-)
C. parapsilosis ATCC 22019	27	-	2	(-)

Note: - and (-): reading or test not done, respectively.

DISCUSSION

Interpretation criteria for susceptibility tests to antimicrobials are currently restricted to reference values for *Candida* spp. by the disk diffusion method and for *Candida* spp. and *C. neoformans* by the broth microdilution method according to CLSI 2008 and 2009 guidelines, respectively (6, 7). Concerning other species, the few studies reported do not define criteria to interpret *in vitro* results and clinical correlations. Preliminary data as reported in the present study are an important contribution to the development or improvement of methods or techniques to characterize *C. laurentti* isolates in the diagnostic clinical laboratory.

A simple and low cost method like disk diffusion is an interesting possibility, which could be applied in modestly supplied laboratories. Studies on physiological characteristics and the profile of responses to antifungals could be useful in a situation concerning, for example, a mycosis caused by low frequency fungi. Studies on environmental fungi are necessary because the environment is where individuals or animals come into contact with microorganisms, and are colonized and infected, but they should also consider related factors such as immunocompromised hosts, microbial load and virulence potential of the microorganism.

In vitro resistance of environmental isolates of *C. laurentii* to antifungals has been reported. Lord et al. (15) showed that eight isolates from bird excreta were resistant to fluconazole. Resistance to fluconazole but susceptibility to other azoles was also described by Bernal-Martinez et al. (3). However, Ferreira-Paim et al. (8) did not detect *in vitro* resistance in 38 isolates, but they did find dose-dependent susceptibility to fluconazole in 71% of cases.

In this study, results obtained by the disk diffusion method, not confirmed by broth dilution, indicated four resistant isolates according to the guidelines issued by CLSI (7). It should be remembered that those interpretative criteria are related to *Candida* spp. and *C. neoformans*. However, it should be noted that most errors detected were MiE and VM. This emphasizes the need to better understand the biology of *C. laurentii* and its *in vitro* behavior and to define the most adequate methodology to study susceptibility to antifungals, and the corresponding criteria for interpretation in order to compare results from different studies. In general the susceptibility of isolates from immunocompromised patients on extended treatment should be determined to be able to detect decreased susceptibility (4).

Minimal fungicide concentrations were only determined for three isolates, which is to be expected, since fluconazole is a fungistatic drug unless in very high concentrations (5).

Barry et al. (1) reported acceptable agreement of results from disk diffusion and broth microdilution with *Candida* spp. isolates. Different times of incubation (24 and 48 hours) were considered by these authors in the methodological comparison. Thus, 24 hours incubation was enough for growth of 94% of isolates

in the disk diffusion method. For the remaining isolates 48 hours were necessary. Comparison between both methods in the present study was limited to a modest concordance due to the small number of samples and lack of standardization of the susceptibility test by disk diffusion for *C. laurentii*. However, Pfaller et al., (20) have shown a good correlation between disk diffusion and broth microdilution for *C. neoformans*. Nevertheless, this is the first work that draws attention to the importance of studies that seek to standardize simple methodologies such as disk diffusion, for studies of yeast uncommon in the clinical laboratory.

The incubation temperature was set to 30ºC allowing faster fungal growth while some isolates did not grow well at 37ºC (3). This possibility contributed to a better control of trailing, which increased with the time of incubation. Trailing is an effect of considerable importance in the disk diffusion method (data not shown) and requires good technical knowledge to develop protocols where its effect is minimal. Other variables like inoculum size and culture media among others should be evaluated to achieve ideal conditions.

Rigorous standardization of interpretation criteria are necessary to obtain *in vitro* reproducible results of susceptibility by the disk diffusion method and highly comparable to reference methods such as broth microdilution. Species-specific criteria should be defined to characterize isolate susceptibility or resistance, which correlate to *in vivo* responses. Alternative methods are available to determine sensitivity profiles to antifungals, but no consensus has been established for the evaluation of non-fermentative yeasts like *Cryptococcus* spp., leading, according to Bernal-Martinez et al. (3) to difficulties in comparing MIC values or characterizing isolates as susceptible, susceptible-dose dependent or resistant.

According to this study, fluconazole may be the antifungal of choice for some isolates of the *C. laurentii* complex but some variables may interfere in the susceptibility testing *in vitro* by the disk diffusion method. These should be known and controlled before inferences on *in vitro* susceptibility or resistance of *C. laurentii* are made. The broth microdilution method seems to provide more consistent results, although it is still necessary to establish interpretation criteria.

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