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SUSCEPTIBILITY TO HEAT AND ANTIFUNGAL AGENTS OF *CRYPTOCOCCUS NEOFORMANS* VAR. *NEOFORMANS* (SEROTYPE D) ISOLATED FROM *EUCALYPTUS* SPP IN RIO GRANDE DO SUL, BRAZIL

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ABSTRACT

In this work we studied the susceptibility to heat and antifungal agents of the first strains of environmental *Cryptococcus neoformans* var *neoformans* (serotype D) isolated in the state of Rio Grande do Sul, Brazil. In order to achieve a rigorous analysis, we employed the methodology recommended by NCCLS, Yeast Nitrogen Base (YNB) proposed by Ghannoum *et al* (YNB-1), Antibiotic medium 3 (AM3) indicated by others, YNB adjusted to the NCCLS methodology (YNB-2) and Etest. Our results indicate that all strains were susceptible to amphotericin B (0.0625 – 0.5 μg/mL), fluconazole (0.125 – 8.0 μg/mL), itraconazole (0.031 – 0.25 μg/mL) and flucytosine (0.125 – 4.0 μg/mL). The *C. neoformans* serotype D strains were more susceptible to heat (47ºC / 30 min) than *C. neoformans* serotype A.

Key words: *Cryptococcus neoformans*, heat susceptibility, antifungal agents

INTRODUCTION

Cryptococcus neoformans, an encapsulated yeast, is the etiological agent of human cryptococcosis. In Brazil, 4.5% of all opportunistic infections in AIDS patients have been reported as being caused by *C. neoformans* (23). Based on physiological and serological differences, *C. neoformans* had been divided in two varieties: *C. neoformans* var. *neoformans* (serotypes A, D, AD) and *C. neoformans* var. *gattii* (serotypes B and C). On the basis of genetic differences it was proposed that *C. neoformans* var. *neoformans* be further subdivided in two varieties: *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *neoformans* (serotype D) (12).

The prevalence of the varieties and serotypes from either clinical or environmental sites differs in accordance with geographical localization. Environmental isolates of C. *neoformans* var. *grubii* from pigeons droppings indicate that serotype A is more common than serotype D in all nations, except Italy, Denmark and Switzerland (8) and have been recovered from approximately 99% of all patients in most countries (6,8,12). The serotype C is rarer than the other serotypes and has never been isolated from the environment. The majority of the few clinical isolates of serotype C have been found in southern California (8). The serotype B was initially isolated from *Eucalyptus camaldulensis* and *E. tereticornis* in Australia and more recently it has been isolated from other trees and immunocompromised (non- AIDS) patients in tropical and subtropical areas (8). In Rio Grande do Sul, *C. neoformans* var. *grubii* (serotype A) has been recovered from AIDS patients and pigeons excreta (1,6,14). The prevalence of *C. neoformans* var. *neoformans* (serotype D) among clinical isolates has ranged from 0 to 100% depending on the region of the world. In Brazil, the prevalence of serotype D is very low (29), and in the state of Rio Grande do Sul there were no

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records of clinical *C. neoformans* var. *neoformans* (serotype D) isolates.

Recently Ribeiro (28) isolated nine *C. neoformans* var. *neoformans* (serotype D) strains from 99 samples of *Eucalyptus* spp. As pointed out by Martinez *et al*. (21), the thermal sensibility of *C. neoformans* var. *neoformans* (serotype D) is a consistent explanation for the geographic differences between serotype A and D, and for the dermatotropism and rhinotropism observed. The purpose of this investigation was to assess the susceptibility of the first environmental *C. neoformans* var. *neoformans* (serotype D) isolates in Rio Grande do Sul, to heat and to a panel of antifungal agents commonly used in the treatment of infections. Because results of susceptibility tests based on M27-A2 methodology have been questioned due to factors such as suboptimal growth in RPMI 1640 medium and narrow amphotericin B MIC ranges (15,20), we decided to compare amphotericin B MICs obtained with RPMI 1640 medium to assays performed using Yeast Nitrogen Base proposed by Ghannoum *et al*. (YNB-1)(13), Antibiotic medium 3 (AM3) and Yeast Nitrogen Base supplemented medium adjusted to the M27-A2 methodology, which we named YNB-2 and the Etest method.

MATERIALS AND METHODS

Isolates

Porto Alegre, the capital city of the South Brazilian state Rio Grande do Sul is situated at 30º S latitude and 51º W longitude. The city is 10 m above the sea level with an average temperature of 22ºC and an annual rainfall of 1118 mm. Ninety-nine samples were collected from different *Eucalyptus* species (Table 1). Twenty other environmental strains of *C. neoformans* serotype A, isolated from pigeon excreta from Porto Alegre, Santa Cruz do Sul and Santa Maria (14), were also included in the analysis of heat susceptibility. All isolates were identified as *C. neoformans* by positive Niger seed agar response, as well as urease test, ability to grow at 35ºC and a negative nitrogen test. The profiles of carbon compound assimilation were also determined (4). Canavanine-glycine-bromothymol blue agar medium, assimilation of D-proline and D-tryptophan were used for differentiation of the two varieties (9) and serotyping was performed by a slide agglutination test (Crypto Check; Iatron Co; Japan).

Table 1. Origin of the environmental isolates of *Cryptococcus neoformans* (serotype D) isolated from *Eucalyptus* spp in Rio Grande do Sul, Brazil.

Location	Number of samples collected	Number of samples identified as *C. neoformans*
Barra do Ribeiro	53	0
Camaquã	8	0
Ijuí	3	0
Novo Hamburgo	2	2
Porto Alegre	7	5
São Leopoldo	2	2
São Lourenço do Sul	4	0
Sertão Santana	7	0
Soledade	3	0
Total	99	9

Susceptibility to heat (21)

A 1-ml aliquot of cells suspended at a density of 10^4 cells per ml in distilled water was incubated at 47ºC in water bath during 30 min. After incubation 20 µL of the cells suspension was plated onto Sabouraud dextrose agar and incubated for 48h at 30ºC. The survival percentage was determined by comparison to non-heat-treated control samples plated onto Sabouraud dextrose agar.

Antifungal susceptibility tests

The antifungal agents used were amphotericin B (Sigma), fluconazole (Pfizer), itraconazole (Jansen) and flucytosine (Sigma).

Test media were: RPMI 1640 (American Biorganics Inc.), indicated by the National Committee for Laboratory Standards (NCCLS) as reference, and Yeast Nitrogen base (Difco) were prepared according to manufacturer instructions. After reconstitution, both media were supplemented with glucose to obtain a final concentration of 0.5% in YNB (13) and 2% in RPMI 1640 (24). Both RPMI 1640 medium and YNB were buffered to pH 7.0 with 3-(N-morpholino) propanesulphonic acid (MOPS; Sigma, St. Louis, Mo, USA) to a final concentration of 165mM (NCCLS). Antibiotic medium 3 (AM3) (BBL) was supplemented with glucose to a final concentration of 2%; the buffering capacity was increased by adding 1g of dipotassium monophosphate per liter and 1g of monopotassium monophosphate per liter and pH was adjusted to 7.0 with NaOH (20). All three media were filter sterilized by using 0.22-µm membrane (Millipore).

The MICs of the tested agents were determined for each isolate in accordance with National Committee Laboratory Standards (NCCLS) macrodilution guidelines (24). The tubes were incubated at 35ºC and were read after 48h of incubation. The MIC of amphotericin B in RPMI 1640, YNB and AM3 was defined as the lowest concentration of drug that resulted in complete inhibition of visible growth. The tests were performed using two techniques with YNB: YNB-1 as indicated by Ghannoum *et al.* (13) and YNB-2 which use the same broth, but using the M27-A2 method (NCCLS). The MIC of azoles and flucytosine in RPMI 1640 were determined according to M27-A method (24). The data were reported as MIC ranges and MICs at which 50% and 90% of these isolates were inhibited. Quality

control testing was performed in accordance with NCCLS document M27-A2. *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019 (24) were used as quality control for the susceptibility tests.

Etest

To prepare the agar plates, the double-strength, filter-sterilized RPMI 1640 with 2% glucose was buffered with potassium phosphate at pH 7.0 and combined with an equal volume of heat-sterilized double-strength agar to yield the correct final concentration of medium in a 1.5% agar gel. The inoculum suspensions of *C. neoformans* strains matching the turbidity of McFarland #1 standard were swabbed onto the surface of the agar plate and allowed to dry for 15 min before the addition of the Etest strip (22). One Etest antimicrobial gradient strip was placed in each Petri dish. The plates were incubated for 48 and 72h, and the MIC was the point at which the zone of complete inhibition intersected the strip. Etest antimicrobial gradient (AB Biodisk, Solna, Sweden) strips containing amphotericin B, fluconazole, itraconazole and flucytosine were employed (32).

RESULTS

All isolates of *C. neoformans* var. *neoformans* (serotype D) grew well in RPMI 1640 broth, YNB-1, YNB-2, Antibiotic medium 3 and RPMI-1640 agar, allowing MICs to be determined after 72h incubation.

Table 2 summarizes the *in vitro* susceptibilities of the nine cultures tested by NCCLS method. The results are reported as MIC ranges, MIC_{50}s (50% of strains were inhibited) and MIC_{90}s (90% of strains were inhibited). A broad range of MICs was observed with fluconazole and flucytosine; more narrow MIC ranges were showed with amphotericin B and itraconazole. Table 3 summarizes the *in vitro* susceptibilities of the isolates to amphotericin B, as determined by NCCLS recommended medium, and other media suggested in the literature. All media employed showed similar MIC ranges, but YNB-1 showed slightly higher results. The MIC_{50} and MIC_{90} were the same for RPMI-1640, AM3 and YNB-2, but again higher for YNB-1. Based on the M27-A2 technique breakpoints, all the isolates were considered to be sensitive to the antifungal agents tested.

The MICs obtained by Etest are shown on Table 4. Itraconazole MICs are narrower in range, but amphotericin B, fluconazole and flucytosine had broad range of MICs. Based on $MIC_{50\%}$ or $MIC_{90\%}$, all the isolates were considered to be sensitive to the antifungal agents tested.

Among the 9 serotype D strains tested, 5 (55.5%) did not survive heat treatment and 4 (44.5%) showed percent survival varying between 22 and 76% when compared to the number of colonies in an equivalent suspension of cells not exposed to heat. When the 20 serotype A strains were assayed, only 3 (15%) did not grow on Sabouraud Dextrose agar after the thermal treatment; the range of percent survival was from 16 to 86%. The average percent survival of serotype D was 23.2%, while for serotype A it was 48%.

Table 3. Comparison of *in vitro* susceptibility of *Cryptococcus neoformans* serotype D strains to amphotericin B using in different media and assay types.

Assay types[a]	MIC[a] (µg/ml)			
	Range	50%[b]	90%[c]	GeoM[b]
RPMI	0.0625-0.5	0.125	0.25	0.134
AM3	0.0625-0.5	0.125	0.25	0.156
YNB-1	0.125-1.0	0.25	0.5	0.269
YNB-2	0.0625-0.5	0.125	0.25	0.170
E-test	0.0625-0.5	0.125	0.5	0.157

[a]See text for details; [b]50% and [c]90%, MICs for 50% and 90% of isolates tested, respectively; [d]GeoM; geometric mean.

Table 2. Susceptibility of *Cryptococcus neoformans* serotype D to antifungal agents using the M27-A2 method.

Antifungal Agents	MIC[a] (µg/ml)			
	Range	50%	90%	GeoM[b]
Amphotericin B	0.0625-0.5	0.125	0.255	0.145
Fluconazole	0.125-8.0	1.0	4.0	1.46
Itraconazole	0.031-0.25	0.125	0.125	0.099
Flucytosine	0.125-4.0	0.5	2.0	0.793

[a]MICs for 50% and 90% of isolates tested; [b]geometric mean.
C. krusei ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control.

Table 4. Susceptibility of *Cryptococcus neoformans* serotype D isolates to antifungal agents determined by using the Etest method.

Antifungal Agents	MIC[a] (µg/ml)			
	Range	50%	90%	GeoM[b]
Amphotericin B	0.0625-0.5	0.125	0.5	0.157
Fluconazole	0.25-8.0	2.0	4.0	1.851
Itraconazole	0.031-0.25	0.125	0.25	0.157
Flucytosine	0.125-4.0	1.0	2.0	0.857

[a]See text for details; [b]50% and [c]90%, MICs for 50% and 90% of isolates tested, respectively; [d]GeoM; geometric mean.

DISCUSSION

An understanding of the epidemiology of cryptococcosis may provide a rational framework for the design of prevention guidelines and more effective therapies. We believe that attempts to correlate specific environmental exposures to cryptococcal strains of known susceptibility profile may help to address some questions (14).

Based on these epidemiological statements, it is important to emphasize that cryptococcosis therapy may become even more difficult due the emergence of antifungal resistance. Amphotericin B (17,19), fluconazole (3,25,26,27), and flucytosine resistance have been well documented (30). Cross-resistance to both azole-types and amphotericin B has also been described (16).

In addition, recent articles reporting primary (intrinsic) resistance of *C. neoformans* var. *neoformans* (serotype D) to flucytosine (18) and, the isolation of fluconazole-resistant *C. neoformans* from an immunocompetent patient, without prior exposure to azoles (25), emphasizes the importance of carrying out susceptibility tests before beginning of therapy. This study reports the susceptibility testing of the first *C. neoformans* serotype D isolated from *Eucalyptus* spp. trees in Rio Grande do Sul state, employing additional methods for more accurate delineation of susceptibility profiles to antifungal agents.

The results of susceptibility tests to antifungal agents reported here are similar to those reported by others authors (7,15). However, some aspects deserve attention. In 2001, we have compared the susceptibility of clinical and environmental *C. neoformans* isolated in southern Brazil and observed that clinical isolates were less susceptible to fluconazole than environmental isolates, all of them of serotype A (1). Here, environmental strains showed a very similar pattern for amphotericin B and azoles, being all strains sensitive. One of the reasons to the absence of azole resistance might be the source of strains, which may have not had previous contact with azoles. The contrary has been reported in Italy, where clinical serotype D is prevalent. Tortorano *et al.* (31) showed that serotype A clinical strains were less susceptible to fluconazole than serotype D clinical isolates. This issue may deserve more attention.

The reference M27-A2 method based on RPMI 1640 medium has been used to test *C. neoformans* isolates (1,2,10,20). In spite of this indication, some previous reports suggested that RPMI 1640 medium did not support suitable growth of *C. neoformans*. In the present study, we have found that this medium, supplemented with 2% glucose, provided adequate growth of all strains tested; glucose supplementation is an alternative mentionated in the M27-A2 method (24). Due to reported concerns regarding amphotericin B resistance, in this study we have tested Antibiotic medium 3 (AM3), as indicated by Lozano-Chiu *et al*. (20), Yeast Nitrogen Base, as recommended by Ghannoum *et al*. (13), and also the same medium with changes in inoculum size and endpoint determination (YNB-2). The results presented have shown that the pattern of susceptibility to amphotericin B obtained with YNB-2, RPMI-1640 medium and AM3 was closely similar, with overlap of MIC ranges. YNB-1 resulted in a broader MIC range, though amphotericin B resistant strains were not detected. These results are similar to those already obtained from clinical strains of *C. neoformans* serotype A using the same media (2).

The MICs to flucytosine were low and so, based on established breakpoints, all strains were considered to be sensitive to this drug. This finding must be interpreted with caution, because approximately 2% of *C. neoformans* isolates are resistant to flucytosine prior to treatment (30). So, we must consider that the number of isolates studied was scarce, and DNA studies have shown that Brazilian *C. neoformans* isolates appeared to be less heterogenous than those isolated from other regions (11).

The Etest method performed on glucose-supplemented RPMI 1640 agar is an excellent method of discrimination between susceptible and resistant strains of *C. neoformans* (20,22,32). The susceptibility pattern obtained by Etest demonstrated a narrow range MICs to amphotericin B, and MIC_{50} and MIC_{90} one log dilution higher than the NCCLS method. For all antifungal agents studied, we have observed no significant changes on MICs by this method. This is in accordance with previous studies, that found complete or a high level of agreement between Etest and NCCLS method (5,22,32). The Etest has been considered an excellent method to distinguish amphotericin-B-resistant yeasts; it is reproducible, much simpler to set up than broth dilution methods and less labour-intensive. Thus, it has been recommended for routine use with amphotericin B and flucytosine (32).

Recently, Martinez *et al*. (21) analysed the heat susceptibility of 19 strains from each serotype group and observed a wide variation; *C. neoformans* serotype D strains being more susceptible. Our finds are in accordance with this study and corroborate what Dromer *et al*. (8) pointed out in that the differences in the prevalence of serotype A and D of *C. neoformans* may reflect climatic tolerances. The isolation of *C. neoformans* var. *neoformans* (serotype D) in Rio Grande do Sul (28), the southernmost and coolest state of Brazil, reflects the characteristics referred above, and might explain the rarity of this serotype in other areas of Brazil with sub-tropical and tropical climates. However, few studies were conducted and data are still scarce in this area (29).

RESUMO

Susceptibilidade de *Cryptococcus neoformans* var. *neoformans* (sorotipo D) isolados de *Eucalyptus* spp., no Rio Grande do Sul (Brasil), frente ao calor e a agentes antifúngicos

Este estudo foi realizado com os primeiros isolados ambientais de C. *neoformans* sorotipo D, obtidos no Rio Grande

do Sul. Objetivando-se avaliar a susceptibilidade a agentes antifúngicos de forma mais rigorosa, utilizou-se a técnica de referência proposta pelo NCCLS, Caldo Yeast Nitrogen Base (YNB) proposto por Ghannoum *et al.*, Antibiotic medium 3, caldo YNB adequado à metodologia do NCCLS e o E-test. Os resultados indicaram que todos os isolados foram sensíveis à anfotericina B (0,0625 -0,5 µg/mL), fluconazol (0,125 – 4,0 µg/mL), itraconazol (0,031 – 0,25 µg/ml) e fluorocitosina (0,125-4,0 µg/mL) através das técnicas empregadas. Nos testes de termotolerância (47ºC/30 min), observou-se que as culturas de *C. neoformans* sorotipo D são mais sensíveis do que as de C. *neoformans* sorotipo A.

Palavras-chave: *Cryptococcus neoformans*, susceptibilidade, antifúngicos

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