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DIFFERENT CULTURE MEDIA APPLIED TO THE STUDY OF *CRYPTOCOCCUS NEOFORMANS* SUSCEPTIBILITY TO AMPHOTERICIN B AND FLUCONAZOLE

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ABSTRACT

This study compared the performance of three culture media used for the determination of minimal inhibitory concentration (MIC) of amphotericin B and fluconazole against 48 *Cryptococcus neoformans* strains. For amphotericin B the media employed were RPMI 1640 medium, as indicated by NCCLS as reference, Yeast Nitrogen Base (YNB) as proposed by Ghannoum *et al*. (YNB-1), Antibiotic medium 3 (AM3) indicated by others, and YNB as proposed by us (YNB-2). In YNB-1, amphotericin B showed broader MIC ranges, varying from 0.125 µg/ml to 1.0 µg/ml; in RPMI 1640 medium and YNB-2 the MICs were similar and in AM3 the MIC ranges were narrower. The same pattern was observed for MIC 90%. For fluconazole the media employed were RPMI 1640 and YNB-1. The MIC ranges were broader and elevated when YNB-1 was employed. MIC 50% were 2.0 µg/ml in RPMI 1640 medium and 4.0 µg/ml in YNB-1. Our results indicate that RPMI 1640 supplemented with 2% glucose allowed a good growth at 48h; YNB as recommended by Ghannoum *et al.* provided a broader MIC range, AM3 provided a narrow MIC range for amphotericin B, and YNB-2 was very similar to RPMI 1640 medium.

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

INTRODUCTION

Cryptococcosis has been the most common life-threatening fungal infection in patients with AIDS, affecting as many as 8% of patients with advanced HIV infection in the United States and as many as 30% of these patients in other parts of the world (3,18).

The minimal inhibitory concentrations (MICs) of amphotericin B and fluconazole against *C. neoformans* can be useful to monitor the possible development of resistance during therapy and to identify primary resistance when lack of response is observed, so that drug therapy type and/or dose can be modified (4). Amphotericin B (8,9,10,17) and fluconazole-*C. neoformans*-resistant strains were detected in antifungal susceptibility tests (1,13,14,15).

The results of susceptibility tests based on M27-A methodology have been questioned because of factors such as suboptimal growth in RPMI 1640 medium and narrow MIC ranges (7,11,16). Since these factors could be masking true *C. neoformans*-resistant strains, alternative methods have been studied.

The purpose of the present study was to compare the susceptibility to amphotericin B of 48 *C. neoformans* strains through the MICs obtained with RPMI 1640 medium, Yeast

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Nitrogen Base proposed by Ghannoum *et al.* (6) (YNB-1), Antibiotic Medium 3 (AM3) and YNB medium with M27-A methodology, which we named YNB-2. The susceptibility to fluconazole was also evaluated, employing RPMI 1640 and YNB-1.

MATERIALS AND METHODS

Isolates

A total of 48 strains were obtained from cerebrospinal fluid (43), blood (3) and sputum (2) from 38 AIDS patients in Hospital Universitario de Santa Maria, Santa Maria, RS, Brazil. Thirty-eight strains were obtained from patients during the initial diagnosis of cryptococcal infection and ten strains were obtained after the patients had been treated with amphotericin B. All isolates were identified as *C. neoformans* by a positive Niger seed agar response, a positive urease test, ability to grow at 37ºC and a negative nitrogen test. The profiles of carbon compound assimilation were also determined.

Antifungal agents

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

Test media

RPMI 1640 medium (American Biorganics Inc.), indicated by NCCLS as reference, and Yeast Nitrogen Base (Difco) were prepared according to manufacturer instructions. After reconstitution, both were supplemented with glucose to obtain a final concentration of 0.5% in YNB (6) and 2% in RPMI 1640 (12). Both RPMI 1640 medium and YNB were buffered to pH 7.0 with 3-(N-morpholino) propanesulfonic acid (MOPS; Sigma, St Louis, Mo, USA) to a final concentration of 165 mM (6,12). 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 the pH was adjusted to 7.0 with NaOH[11]. All three media were filter sterilized by passage through a 0.22-µm-pore-size filter system (Millipore).

Antifungal susceptibility testing

The MICs of the test agents were determined for each test isolate in accordance with National Committee for Clinical Laboratory Standards (NCCLS) macrodilution guidelines (12). The tubes were incubated at 35ºC and were read after 48h of incubation. The MIC of amphotericin B in RPMI 1640, YNB or in AM3, was defined as the lowest concentration of drug which resulted in complete inhibition of visible growth. The tests were performed using two techniques with YNB broth: YNB-1 as proposed by Ghannoum *et al.* (6) and YNB-2 which was the same broth but applied to M27-A methodology (12). The MIC of fluconazole in RPMI 1640 was defined as the lowest concentration of the drug which resulted in an 80% reduction of fungal growth compared to control. The MIC of fluconazole in YNB-1 was defined as the lowest concentration of drug which resulted in a 50% reduction of fungal growth compared to control; the inoculum in this medium was 1 x 10^4 cfu/ml; the tubes were incubated at 35ºC and were read after 48h of incubation (6). MIC endpoint discrepancies of no more than two dilutions (two tubes) were used to calculate percent agreement with MIC obtained from RPMI 1640 reference medium. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 were used as quality control for the susceptibility tests. The data were reported as MIC ranges and MICs at which 50% and 90% of this isolates were inhibited.

RESULTS

All 48 isolates of *C. neoformans* grew well in RPMI 1640 medium, YNB-1, YNB-2 and Antibiotic Medium 3, allowing MICs to be determined after 48h of incubation. Table 1 summarizes the *in vitro* susceptibilities of the 48 clinical isolates to amphotericin B as determined by the NCCLS recommended medium and others also suggested in the literature. For amphotericin B, MIC ranges were slightly broader with YNB-1 than with YNB-2 and RPMI 1640; AM3 provided the narrowest MIC ranges, and the same was observed with MIC 90%. The MIC 50% was the same for RPMI, YNB-2 and AM3 but one dilution higher for YNB-1. The MIC geometric means obtained were YNB-1 >YNB-2 >RPMI > AM3, and the percent agreement based on RPMI reference medium was 85% with YNB-2, 82% with AM3 medium and 74% with YNB-1. All strains tested were susceptible to low concentrations of amphotericin B whose MICs ranged from 0.0625 to 1.0 µg/ml.

Table 1. Comparison of *in vitro* susceptibility of 48 *C. neoformans* strains to amphotericin B applied in different media.

Media	MIC (µg/ml)[b]			GeoM[c]	% Agreement[d]
	Range	50%	90%		
RPMI[a]	0.0625 - 0.5	0.125	0.25	0.117	-
AM3	0.0625 - 0.25	0.125	0.125	0.109	82
YNB-1[e]	0.125 - 1.0	0.25	0.5	0.186	74
YNB-2[f]	0.0625 - 0.5	0.125	0.25	0.134	85

[a] NCCLS M27-A reference macrodilution method visually after 48h incubation; [b] 50% and 90%, MICs for 50% and 90% of isolates tested, respectively; [c] GeoM; geometric mean; [d] Percent agreement within 2log 2 dilutions of the reference MIC; [e] Yeast Nitrogen Base broth proposed by Ghannoum[6]; [f] Yeast Nitrogen Base broth proposed by Ghannoum adjusted to M27-A methodology.

For the *in vitro* susceptibility tests of fluconazole the MIC ranges changed from 0.5-16 µg/ml in RPMI 1640 medium and from 0.25 to 16 mg/ml in YNB-1 (Table 2). The MIC 50% demonstrated the same variations but the MIC 90% was equal for the two media employed.

Table 2. Comparison of *in vitro* susceptibility of 48 *C. neoformans* strains to fluconazole applied in two different media.

Media	MIC (µg/ml)[b]			GeoM[c]
	Range	50%	90%	
RPMI[a]	0.5 -16	2.0	8.0	1.86
YNB-1[d]	0.25-16	4.0	8.0	1.89

[a] NCCLS M27-A reference macrodilution method visually after 48h incubation; [b] 50% and 90%, MICs for 50% and 90% of isolates tested, respectively; [c] GeoM; geometric mean; [d] Yeast Nitrogen Base broth proposed by Ghannoum[6].

DISCUSSION

In vitro susceptibility tests have been recently standardized (NCCLS M27-A document) for yeasts and intensive investigations are currently underway (2,5,11).

The reference M27-A methodology based on RPMI 1640 medium has been used successfully to test *C. neoformans* isolates (5,7,11). There has been concern regarding the use of the NCCLS method for testing *Cryptococcus neoformans* because some isolates may grow slowly in RPMI 1640 and the recommended incubation time of 72h has been deemed too long for practical use in the clinical laboratory (7,11).

For amphotericin B, some authors suggest that RPMI 1640 medium may not be a good culture medium to warrant good *C. neoformans* growth, thus hindering the detection of resistance (4). In the present study we used RPMI 1640 medium supplemented with 2% glucose and all strains showed good growth at 48h. Glucose supplementation is an alternative mentioned in the M27-A methodology.

The supplemented YNB proposed by Ghannoum *et al.* (6) (YNB-1) has been indicated as a better choice than RPMI 1640 medium for testing amphotericin B, fluconazole and flucytosine. In this study we employed YNB as recommended by Ghannoum *et al.* (6) and also the same medium with changes in inoculum size and endpoint determination (YNB-2). Lozano-Chiu *et al.* (11) found that only AM3 permitted consistent detection of resistant isolates when testing was performed in broth by the M27-A method (12). Our results demonstrate that the pattern of susceptibility to amphotericin B obtained with YNB-2, RPMI 1640 medium and AM3 was closely similar, with a significant overlap in the MIC ranges. In contrast while YNB-1 provided a broader MIC range than the other media, its percent agreement with RPMI 1640 reference medium was poor (74%). The precise meaning of this finding is unknown because we did not employ amphotericin B-resistant *C. neoformans* as control, but this is in accordance with the results of Ghannoum *et al.* (6) and different from those reported by Lozano-Chiu *et al.* (11) who elected AM3 as the best medium. The disagreement with RPMI 1640 medium may indicate the better performance of YNB-1 providing more elevated MICs and thus detecting amphotericin B-resistant – *C. neoformans* strains more properly. The YNB-2 proposed by us showed MICs similar to RPMI 1640 medium.

Fluconazole has been shown to be an effective alternative to amphotericin B in the treatment of cryptococcal meningitis and is the most commonly used antifungal agent in maintenance therapy of this disease (18). The *C. neoformans* susceptibility to fluconazole could be an important predictor of treatment success and MICs can be useful to monitor possible development of resistance during therapy and to identify primary resistance (1,3,4,13,14,15,19).

Table 2 shows that YNB-1 provided higher MICs than RPMI 1640 medium. However, the MIC ranges with YNB-1 were broader than with RPMI 1640 medium. The MIC 50% and MIC geometric means showed the same pattern, with YNB-1> RPMI 1640, but MICs 90% was the same for all media used.

Our results agree with others indicating that YNB-1 medium proposed by Ghannoum *et al.* (6) is superior to the reference method for susceptibility testing of *C. neoformans* (16).

In conclusion, it seems clear that YNB as proposed by Ghannoum *et al.* (6) represents an important alternative medium for *C. neoformans* susceptibility tests, as already postulated by the M27-A document (12). The use of YNB as proposed by us (YNB-2) does not display advantages because its results were very similar to those obtained with the RPMI 1640 reference medium.

RESUMO

Diferentes meios de cultivo empregados ao estudo da suscetibilidade de *Cryptococcus neoformans* frente a anfotericina B e fluconazol

Comparou-se o desempenho de três meios de cultura na determinação das concentrações inibitórias mínimas (CIMs) de dois antifúngicos frente a 48 amostras de *C. neoformans*. Para anfotericina B, os meios empregados foram o Caldo RPMI 1640, indicado como referência pelo NCCLS, o Yeast Nitrogen Base (YNB-1) indicado por Ghannoum *et al.*, o Antibiotic Medium 3 (AM3) utilizado por vários autores e o YNB-2, sugerido por nós, que é o mesmo YNB-1, todavia, adequado à técnica preconizada pelo NCCLS. Para a anfotericina B, a mais ampla faixa de CIMs (0,125-1,0 mg/ml) foi obtida no YNB-1; os intervalos de CIMs obtidos no caldo RPMI 1640 e no YNB-2 foram muito similares enquanto que o AM3 evidenciou

pequenas variações nas CIMs. Para os testes com o fluconazol utilizou-se o RPMI 1640 e o YNB-1; novamente, o mais amplo intervalo de CIMs, assim como as CIMs mais elevadas, foram observados no YNB-1; as variações de CIM 50% foram de 2,0 mg/ml no RPMI 1640 e 4,0 mg/ml no YNB-1. Os resultados obtidos permitem concluir que: o RPMI 1640 suplementado com dextrose 2% permite um bom crescimento em 48h de incubação; o YNB recomendado por Ghannoum *et al*. fornece faixas de CIMs mais amplas; que o AM3 forneceu estreito intervalo de CIMs para a anfotericina B e que o YNB-2 utilizado nos testes com anfotericina B, evidenciou resultados muito semelhantes aos do caldo RPMI 1640.

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

REFERENCES

1. Armengou, A.; Pocar, C.; Mascaró, J.; Garcia-Bragado, F. Possible development of resistance to fluconazole during suppresive therapy for AIDS-associated cryptococcal meningitis. *Clin. Infect. Dis.*, 23(6): 1337-1338, 1996.
2. Casadevall, A.; Perfect, J.R. *Cryptococcus neoformans*. ASM, Washington, 1998, 541p.
3. Coker, R.J.; Harris, J.R.W. Failure of fluconazole treatment in cryptococcal meningitis despite adequate CSF levels. *J. Infect.*, 23: 101-102, 1991.
4. Espinel-Ingroff, A. Clinical relevance of antifungal resistance. *Inf. Dis. Clin. North America*, 11(4): 929-944, 1997.
5. Franzot, S.P.; Hamdan, J.S. In vitro susceptibilities of clinical and environmental isolates of *Cryptococcus neoformans* to five antifungal agents. *Antimicrob Agents Chemother*., 40: 822-824, 1996.
6. Ghannoum, M.A.; Ibrahim, A.S.; Fu, Y.; Schafiq, M.; Edwards, J.E.; Criddle, R.S. Susceptibility testing of *Cryptococcus neoformans*: a microdilution technique. *J. Clin. Microbiol.*, 30: 2881-2886, 1992.
7. Jessup, C.J.; Pfaller, M.A.; Messer, S.A.; Zhang, J.; Tumberland, M.; Mbidde, E.K.; Ghannoum, M.A. Fluconazole susceptibility testing of *Cryptococcus neoformans*: comparison of two broth microdilution methods and clinical correlates among isolates from Ugandan AIDS patients. *J. Clin. Microb.*, 36(10): 2874-2876, 1998.
8. Joseph-Horne, T.; Hollomon, D.; Loeffler, R.S.T.; Kelly, S.L. Cross-resistance to polyene and azole drug in *Cryptococcus neoformans*. *Antimicrob. Agents Chemother.*, 39: 1526-1529, 1995.
9. Joseph-Horne, T.; Loeffler, R.S.T.; Hollomon, D.W.; Kelly, S.L. Amphotericin B resistant isolates of *Cryptococcus neoformans* without alteration in sterol biosynthesis. *J. Med. Vet. Mycol*., 34: 223-225, 1996.
10. Kelly, S.L.; Lamb, D.C.; Taylor, M.; Corran, A.J.; Baldwin, B.C.; Powderly, W.G. Resistance to amphotericin B associated with defective sterol $\triangle$ 8.7 isomerase in a *Cryptococcus neoformans* strains from AIDS patient. FEMS *Microbiol. Lett.*, 122: 39-42, 1994.
11. Lozano-Chiu, M.; Paetznick, V.L.; Ghannoum, M.A.; Rex, J.H. Detection of resistance to amphotericin B among *Cryptococcus neorformans* clinical isolates: performances of three different media assessed by using E-test and National Committee for Clinical Laboratory Standards M27-A Methodologies. *J. Clin. Microbiol.*, 36(10): 2817-2822, 1998.
12. *NATIONAL COMMITTEE OF CLINICAL LABORATORY STANDARDS*. 1997. Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard M27ª National Committee for Clinical Laboratory Standards, Wayne, Pa.
13. Orni-Wasserlauf, R.; Izkhakov, E.; Siegman-Igray, Y.; Bash, E.; Polacheck, I.; Giladi, M. Fluconazole-resistant *Cryptococcus neoformans* isolated from an immunocompetent patient without prior exposure to fluconazole. *Clin. Infect. Dis.*, 29: 1592-1593, 1999.
14. Paugam, A.; Dupoy-Camet, J.; Blanche, P.; Gangneux, J.P.; Tourte-Schaefer, C.; Sicard, D. Increased fluconazole resistance of *Cryptococcuss neoformans* isolated from a patient with AIDS and recurrent meningitis. *Clin. Infect. Dis.*, 19: 976-977, 1994.
15. Peetermans, W.; Bobbaers, H.; Verhaegen, J.; Vandepitte, J. Fluconazole-resistant *Cryptococcus neoformans* var. *gatti* in an AIDS patient. *Acta Clin. Belg.*, 48: 405-409, 1993.
16. Pfaller, M.A.; Rex, J.H.; Rinaldi, M.G. Antifungal susceptibility testing: technical advances and potential clinical applications. *Clin. Infect. Dis*., 24: 776-784, 1997.
17. Powderly, W.G.; Keath, E.A.; Sokol-Anderson, M. Amphotericin B-resistant *Cryptococcus neoformans* in a patient with AIDS. *Infect. Dis. Clin. Pract*., 1: 314-316, 1990.
18. Powderly, W.G. Cryptococcal meningitis in HIV-infected patients. *Curr. Infect. Dis. Reposts*, 2: 352-357, 2000.
19. Witt, M.D.; Lewis, R.J.; Larsen, R.A.; Milefchik, E.N.; Leal, M.A.; Haubrich, R.H.; Richie, J.A.; Edwards, J.E.; Ghannoum, M.A. Identification of patients with acute AIDS-associated cryptococcal meningitis who can be effectively treated with fluconazole: the role of antifungal susceptibility testing. *Clin. Infect. Dis.*, 22: 322-328, 1996.