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ANTIFUNGAL SUSCEPTIBILITIES OF CLINICAL AND ENVIRONMENTAL ISOLATES OF *Cryptococcus neoformans* IN GOIÂNIA CITY, GOIÁS, BRAZIL

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SUMMARY

We evaluated the antifungal activities of amphotericin B, fluconazole, itraconazole and voriconazole in 70 *Cryptococcus neoformans* strains obtained from cerebrospinal fluid from AIDS patients and 40 *C. neoformans* strains isolated from the environment. Four clinical isolates were identified as *C. neoformans* var. *gattii*. The susceptibility test was done using a broth microdilution method according to NCCLS M27-A2. Range minimal inhibitory concentrations (MICs) for *C. neoformans* clinical isolates were 0.06-1.0 µg/mL for amphotericin B, 0.125-8 µg/mL for fluconazole, 0.03-0.5 µg/mL for itraconazole and 0.03-0.25 µg/mL for voriconazole. *C. neoformans* environmental isolates showed range MICs 0.015-0.125 µg/mL, 0.25-2.0 µg/mL, 0.007-0.125 µg/mL and 0.03-0.25 µg/mL for amphotericin B, fluconazole, itraconazole and voriconazole respectively. The MICs results obtained from clinical and environmental isolates showed similar pattern of susceptibility and no resistance has been found in our isolates.

KEYWORDS: *Cryptococcus neoformans*; Antifungal agents; Cerebrospinal fluid; Environment.

INTRODUCTION

Cryptococcus neoformans is an encapsulated, spherical yeast of which three varieties are recognized, *C. neoformans* var. *neoformans* (serotypes D and AD), *C. neoformans* var. *grubii* (serotype A) and *C. neoformans* var. *gattii* (serotypes B and C). The *neoformans* and *grubii* varieties have a worldwide distribution, and it is associated with soil contaminated with bird droppings. *C. neoformans* var. *neoformans* is generally responsible for cryptococcosis in immunocompromised patients, particularly in HIV infected individuals[1,6,22,24]. *C. neoformans* var. *gattii* is geographically restricted to tropical and subtropical climates, and it is most commonly found in association with *Eucalyptus camaldulensis* and *E. tereticornis* trees[5,13,16]. This variety causes infection in apparently healthy hosts and it is considered as a true pathogen[3,11,27]. *C. neoformans* infection occurs after inhalation from environmental sources, of yeast cells into the lung, with hematogenous dissemination to the central nervous system, where causes cryptococcal meningitis, the most common and serious cryptococcal disease manifestation[26].

Until now, the currently accepted therapies for cryptococcosis are limited to amphotericin B, 5-fluorocytosine and fluconazole[4]. However, the treatment drugs for cryptococcosis are not satisfactory, because of their toxicity, their limited ability to clear infections completely, accompanied by the development of resistance in fungi[1]. Thereby, methods for testing the antifungal susceptibility of *C. neoformans* should become important tools for the selection and monitoring of appropriate antifungal drugs for the treatment and prophylaxis of cryptococcal infections.

By the fact that there are few comparative studies carried out on antifungal susceptibilities from environmental and clinical *C. neoformans* isolates, the purpose of this investigation was to verify the *in vitro* susceptibility of 110 *C. neoformans* isolates (70 clinical and 40 environmental) towards four antifungal agents, including fluconazole, itraconazole, voriconazole and amphotericin B, using the broth microdilution method[19].

MATERIALS AND METHODS

1. Organisms: A total of 110 *C. neoformans* isolates were included in this study. They comprised 70 clinical strains isolated from cerebrospinal fluid from AIDS patients and 40 environmental isolates from pigeon excreta and from *Eucalyptus* trees, originated from Goiânia city, located in Midwest region of Brazil. *C. neoformans* was identified by colonial and cellular morphologies and physiological and biochemical characteristics[10]. The varieties were determined in canavanine-glycine-bromothymol blue agar (CGB)[12]. These isolates were stored at 4 ºC on Sabouraud dextrose agar slants (Difco) and subcultured 72 h prior the test.

2. Antifungal susceptibility testing: Standard broth microdilution method recommended by the NCCLS M27-A2[12] was used.

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2.1. Antifungal agents: Amphotericin B (Fungizone, Squibb, USA), itraconazole (Jansen Pharmaceuticals, Beerse, Belgium) and voriconazole (Pfizer Pharmaceutical Group) were dissolved in dimethyl sulfoxide, and fluconazole (Pfizer International, New York, USA) was dissolved in distilled water. Further dilutions of each antifungal agent were prepared with RPMI 1640 medium (Sigma) containing L-glutamine without sodium bicarbonate, and buffered to a pH 7.0 with 0.165M morpholinepropanesulfonic acid (MOPS) (Sigma).

2.2. Inoculum: The suspension of yeast from 48-h-old cultures was prepared in sterile saline (0.85%) adjusted with a spectrophotometer to a cell density of 0.5 McFarland standard at a wavelength of 530 nm. This suspension was diluted at 1:50 followed by a 1:20 dilution in RPMI 1640 in order to obtain a final concentration of 1 x 10^3 to 5 x 10^3 CFU/mL.

2.3. Susceptibility testing: Microtitre plates were covered with 100 µL of different concentrations of the antifungal agents and added with 100 µL of the yeast suspension. A final inoculum of 0.5 x 10^3 to 2.5 x 10^3 CFU/mL and the final concentrations of the antifungal agents ranged from 0.03 to 64 µg/mL for fluconazole and 0.007 to 16 µg/mL for itraconazole, voriconazole and amphotericin B. These plates were incubated at 35 ºC and read after 72 hours. The MIC end points were defined for amphotericin B as the lowest concentration of drug which resulted in a complete inhibition of visible growth, while for the three azoles were defined as the lowest concentration of drug that produced a 80% reduction in fungal growth compared to that one of drug-free growth control. All susceptibility tests were performed twice by each antifungal agent.

3. Quality control: Quality control organism *Candida parapsilosis* ATCC 22019 was included on each day of testing to check the accuracy of the drug dilutions and the reproducibility of the results. The purity and viability of all tested organisms were checked subculturing the inoculum suspension in Sabouraud dextrose agar (Difco).

As the breakpoint susceptibility values have not yet been proposed by the NCCLS M27-A2[12] for *C. neoformans*, the resistance to antifungal agents considered for fluconazole was ≥ 64 µg/mL, for itraconazole and voriconazole ≥ 1 µg/mL and ≥ 2 µg/mL for amphotericin B[14,17,20, 24].

RESULTS

From the 70 clinical isolates obtained from AIDS patients cerebrospinal fluid, 66 were identified as *C. neoformans* var. *neoformans* and four as *C. neoformans* var. *gattii*. All environmental isolates were identified as *C. neoformans* var. *neoformans*.

All *C. neoformans* isolates were susceptible to amphotericin B, fluconazole, itraconazole and voriconazole. However, MIC values of the clinical isolates for all tested antifungal drugs were higher than for the environmental isolates. The analysis of MIC_{50} and MIC_{90} values showed that the major difference between clinical and environmental isolates was verified for amphotericin B. MIC_{50} values for clinical isolates were fourfold higher than that for environmental isolates for this drug.

The MIC ranges and MIC required to inhibit 50% and 90% for amphotericin B, fluconazole, itraconazole and voriconazole are summarized in Table 1. The MICs for organism control *C. parapsilosis* ATCC 22019 tested in the experiments were consistently in agreement with those from the NCCLS reference results.

The MICs of amphotericin B, itraconazole and voriconazole for four clinical isolates of *C. neoformans* var. *gattii* showed low values, while for fluconazole the range MICs was 4-8 µg/mL. The results of MIC values for each *C. neoformans* var. *gattii* are presented in Table 2.

DISCUSSION

Although cryptococcosis is the sixth most common opportunistic infection among HIV positive patients in Brazil[8], little is known of this disease in Goiânia City, State of Goiás.

The varieties in this study were determined in CGB medium and *C. neoformans* var. *neoformans* predominated in isolates from environmental and clinical sources followed by *C. neoformans* var. *gattii*. Previous reports has shown *C. neoformans* var. *neoformans* as prevalent in Brazil[7,9,21]. Interestingly, a number of epidemiological studies have demonstrated that almost all cryptococcal infections in AIDS patients are due to the *neoformans* variety, that has long been known to be associated with pigeon excreta[6,22]. Limited exposure of HIV-infected patients to *C. neoformans* var. *gattii* can result in fewer infections by this variety. In spite of seeking *C. neoformans* var. *gattii* in environment, including eucalyptus trees, we are unable to isolate it.

The *in vitro* susceptibilities of *C. neoformans* strains to different antifungal drugs have been studied by a number of investigators[2,15,18,24]. However, there is few comparison of MIC data between clinical and environmental isolates. Our results showed similarity in the pattern of susceptibility in concern to the origin of the isolates (clinical or environmental). Testing antifungal susceptibility for environmental isolates may serve to evaluate the patterns of susceptibility of clinical isolates from the same geographic areas. The exposure to *C. neoformans* isolates could be associated with the infection risk in a given population.

In spite of fluconazole being the drug that commands maintenance treatment protocols for AIDS patients, in the present study, the highest MICs were detected for this drug. Other reports have demonstrated lower *in vitro* activity of fluconazole compared with other azoles[4,23]. SOARES *et al.*[24] by using the EUCAST broth microdilution method, obtained *C. neoformans* isolates from pigeons resistant to fluconazole. The lowest activity *in vitro* and the highest one *in vivo* can be explained by plasmatic levels of fluconazole that can be higher that other azoles as itraconazole[18].

Although all the tested isolates have been susceptible to fluconazole, *C. neoformans* var. *gattii* isolates exhibited relatively higher MIC values than *C. neoformans* var. *neoformans*. Interestingly, infections due to *C. neoformans* var. *gattii* often require prolonged antifungal therapy[24].

As it was seen in Tables 1 and 2, our MIC ranges obtained for amphotericin B allow us to conclude that the studied clinical and environmental isolates are susceptible to this drug. Amphotericin B is considered the treatment of choice for the initial stages of therapy and it is known that reports about amphotericin B resistance *C. neoformans* isolates are scarce.

Table 1
Antifungal activities of amphotericin B and azoles derivatives against 70 clinical and 40 environmental isolates of *C. neoformans* as determined by broth microdilution method

Antifungal agents and strain group*	Minimal Inhibitory Concentration (µg/mL)		
	Range	MIC_{50}	MIC_{90}
Amphotericin B			
clinical	0.06-1.0	0.5	0.5
environmental	0.015-0.125	0.06	0.125
Fluconazole			
clinical	0.125-8	2.0	4.0
environmental	0.25-2.0	1.0	1.0
Itraconazole			
clinical	0.03-0.5	0.06	0.125
environmental	0.007-0.125	0.03	0.125
Voriconazole			
clinical	0.03-0.25	0.06	0.125
environmental	0.03-0.25	0.06	0.125

*Clinical isolates: *C. neoformans* var. *neoformans* n = 66; *C. neoformans* var. *gattii* n = 4; Environmental isolates: *C. neoformans* var. *neoformans* n = 40

Table 2
Antifungal activities of amphotericin B and azoles derivatives against *C. neoformans* var. *gattii* clinical isolates as determined by broth microdilution method

Isolates	Minimal Inhibitory Concentration (µg/mL)			
	Amphotericin B	Fluconazole	Itraconazole	Voriconazole
L1	0.25	4.0	0.125	0.5
L9	0.125	4.0	0.125	0.125
L20	0.25	8.0	0.06	0.25
L48	0.25	4.0	0.25	0.06

Besides fluconazole, products currently available, voriconazole and itraconazole, appear to represent interesting alternatives for AIDS patients maintenance treatment. Voriconazole and itraconazole demonstrate excellent *in vitro* activity against *C. neoformans*[23]. As showed in Table 1, all strains were highly sensitive to two azoles; 90% of the isolates showed a minimum inhibitory concentration of 0.125 µg/mL for the two antifungal agents.

In conclusion although no resistance has been found in our isolates, we continue paying attention to the emerging resistance to azoles, mainly fluconazole which should be searched, due to the widespread use of fluconazole as primary prophylaxis in AIDS patients.

RESUMO

Suscetibilidade antifúngica de isolados clínicos e ambientais de *Cryptococcus neoformans* na cidade de Goiânia, Goiás, Brasil

A atividade antifúngica de anfotericina B, fluconazol, itraconazol e voriconazol foi avaliada em 70 amostras de *Cryptococcus neoformans* isoladas de liquido céfalo raquidiano (LCR) de pacientes com AIDS e em 40 amostras de *C. neoformans* obtidas do meio ambiente. Dentre os isolados 66 foram identificados como *C. neoformans* var. *neoformans* e quatro isolados clínicos, como *C. neoformans* var. *gattii*. Para a realização dos testes de suscetibilidade foi utilizado o método de microdiluição em meio líquido segundo o NCCLS M27-A2. As concentrações inibitórias mínimas (CIMs) para os isolados clínicos variaram de 0,06-1,0 µg/mL para anfotericina B, 0,125-8 µg/mL para fluconazol, 0,03-0,5 µg/mL para itraconazol e 0,03-0,25 µg/mL para voriconazol, enquanto que para as amostras ambientais de *C. neoformans* as concentrações inibitórias variaram de 0,015-0,125 µg/mL, 0,25-2,0 µg/mL, 0,007-0,125 µg/mL e 0,03-0,25 µg/mL para anfotericina B, fluconazol, itraconazol e voriconazol, respectivamente. Os resultados das concentrações inibitórias mínimas obtidas para os isolados clínicos e ambientais mostraram semelhança com relação ao perfil de suscetibilidade, não tendo sido encontrados isolados resistentes a nenhum dos antifúngicos, levando-se em consideração a metodologia e critério de interpretação estudados.

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REFERENCES

1. CASADEVALL, A. & PERFECT, J.R. - ***Cryptococcus neoformans*.** Washington, American Society for Microbiology, 1998.

2. CHANDENIER, J.; ADOU-BRYN, K.D.; DOUCHET, C. *et al.* - *In vitro* activity of amphotericin B, fluconazole and voriconazole against 162 *Cryptococcus neoformans* isolates from Africa and Cambodia. **Europ. J. clin. Microbiol. infect. Dis., 23:** 506-508, 2004.

3. CHEN, S.; SORRELL, T.; NIMMO, G. *et al.* - Epidemiology and host- and variety-dependent characteristics of infection due to *Cryptococcus neoformans* in Australia and New Zealand. Australasian Cryptococcal Study Group. **Clin. infect. Dis., 31:** 499-508, 2000.

4. DATTA, K.; JAIN, N.; SETHI, S. *et al.* - Fluconazole and itraconazole susceptibility of clinical isolates of *Cryptococcus neoformans* at a tertiary care centre in India: a need for care. **J. Antimicrob. Chemother., 52:** 683-686, 2003.

5. ELLIS, D. & PFEIFFER, T.J - Natural habitat of *Cryptococcus neoformans* var. *gattii*. **J. clin Microbiol., 28:** 1642-1644, 1990.

6. FERNANDES, O.F.L.; COSTA, T.R.; COSTA M.R. *et al*. - *Cryptococcus neoformans* isolados de pacientes com AIDS. **Rev. Soc. bras. Med. trop., 33:** 75-78, 2000.

7. FRANZOT, S.P. & HAMDAN, J.S. - *In vitro* susceptibilities of clinical and environmental isolates of *Cryptococcus neoformans* to five antifungal drugs. **Antimicrob. Agents Chemother., 40:** 822-824, 1996.

8. GUIMARÃES, M.D.C. - Estudo temporal das doenças associadas à AIDS no Brasil, 1980-1999. **Cad. Saúde públ. (Rio de J.), 16** (suppl. 1): 21-36, 2000.

9. HORTA, J.A.; STAATS, C.C.; CASALI, A.K. *et al.* - Epidemiological aspects of clinical and environmental *Cryptococcus neoformans* isolates in Brazilian State Rio Grande do Sul. **Med. Mycol., 40:** 565-571, 2002.

10. KURTZMAN, C.P. & FELL, J.W. - **The yeasts, a taxonomic study**. 4. ed. Amsterdam, Elsevier, 1998.

11. KWONG-CHUNG, K.J. - *Cryptococcus neoformans*. In: KWONG-CHUNG, K.J. & BENNETT, J.E. **Medical Mycology**. Philadelphia, Lea & Febiger, 1992. p. 397-466.

12. KWONG CHUNG, K.J.; POLACHECK, I. & BENNETT, J.E. - Improved diagnostic medium for separation of *Cryptococcus neoformans* var. *neoformans* (serotypes A and D) and *Cryptococcus neoformans* var. *gattii* (serotypes B and C). **J. clin. Microbiol., 15:** 535-537, 1982.

13. LICEA, B.A.; GARZA, D.G.; URBIETA, V.F. & OLIVARES, R.A.C. - Aislamiento y caracterización de *Cryptococcus neoformans* var. *gattii* a partir de muestras de *Eucalyptus camaldulensis* en la ciudad de México. **Rev. iberoamer. Micol., 16:** 40-42, 1999.

14. LOZANO-CHIU, M.; PAETZNICK, V.L.; GHANNOUM, M.A. & REX, J.H. - Detection of resistance to amphotericin B among *Cryptococcus neoformans* 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:** 2817-2822, 1998.

15. MAXWELL, M.J.; MESSER, S.A.; HOLLIS, R.J.; DIEKEMA, D.J. & PFALLER, M.A. - Evaluation of Etest method for determining voriconazole and amphotericin B MICs for 162 clinical isolates of *Cryptococcus neoformans*. **J. clin. Microbiol., 41:** 97-99, 2003.

16. MONTENEGRO, H. & PAULA, C.R. - Environmental isolation of *Cryptococcus neoformans* var. *gattii* and *Cryptococcus neoformans* var. *neoformans* in the city of São Paulo, Brazil. **Med. Mycol., 38:** 385-390, 2000.

17. MONDON, P.; PETTER, R.; AMALFITANO, G. *et al.* - Heteroresistance to fluconazole and voriconazole in *Cryptococcus neoformans*. **Antimicrob. Agents Chemother., 43:** 1856-1861, 1999.

18. MORAES, E.M.; PRÍMOLA, N.S. & HAMDAN, J.S. - Antifungal susceptibility of clinical and environmental isolates of *Cryptococcus neoformans* to four antifungal drugs determined by two techniques. **Mycoses, 46:** 164-168, 2003.

19. NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS - Reference method for broth dilution antifungal susceptibility testing of yeasts: approved standard. Villanova, NCCLS, 2002. v. 17, No. 9. (Document M27-A2).

20. NGUYEN, M.H. & YU, C.Y. - *In vitro* comparative efficacy of voriconazole and itraconazole against fluconazole-susceptible and -resistant *Cryptococcus neoformans* isolates. **Antimicrob. Agents Chemother., 42:** 471-472, 1998.

21. NISHIKAWA, M.M.; LAZERA, M.S.; BARBOSA, G.G. *et al.* - Serotyping of 467 *Cryptococcus neoformans* isolates from clinical and environmental sources in Brazil: analysis of host and regional patterns. **J. clin. Microbiol., 41:** 73-77, 2003.

22. OHKUSU, M.; TANGONAN, N.; TAKEO, K. *et al*. - Serotype, mating type and ploidy of *Cryptococcus neoformans* strains isolated from patients in Brazil. **Rev. Inst. Med. trop. S. Paulo, 44:** 299-302, 2002.

23. PFALLER, M.A.; ZHANG, J. & MESSER, S.A. - *In vitro* activities of voriconazole, fluconazole, and itraconazole against 566 clinical isolates of *Cryptococcus neoformans* from the United States and Africa. **Antimicrob. Agents Chemother., 43:** 169-171, 1999.

24. SOARES, M.C.B.; PAULA, C.R.; DIAS, A.L.T.; CASEIRO, M.M. & COSTA, S.O.P. - Environmental strains of *Cryptococcus neoformans* variety *grubii* in the city of Santos, SP, Brazil. **Rev. Inst. Med. trop. S.Paulo, 47:** 31-36, 2005.

25. SORRELL, T.C. - *Cryptococcus neoformans* variety *gattii*. **Med. Mycol., 39:** 155-168, 2001.

26. STEENBERGEN, J.N. & CASADEVALL, A. - The origin and maintenance of virulence for the human pathogenic fungus *Cryptococcus neoformans*. **Microbes Infect., 5:** 667-675, 2003.

27. TAYLOR, M.B.; CHADWICK, D. & BARKHAM, T. - First reported isolation of *C. neoformans* var. *gattii* from a patient in Singapore. **J. clin. Microbiol., 40:** 3098-3099, 2002.

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