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Original article

In vitro antifungal susceptibility of clinical and environmental isolates of *Aspergillus fumigatus* and *Aspergillus flavus* in Brazil

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ABSTRACT

The *in vitro* susceptibility of 105 clinical and environmental strains of *Aspergillus fumigatus* and *Aspergillus flavus* to antifungal drugs, such as amphotericin B, azoles, and echinocandins was evaluated by the broth microdilution method proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Following the EUCAST-proposed breakpoints, 20% and 25% of the clinical and environmental isolates of *A. fumigatus*, respectively, were found to be resistant to itraconazole (Minimal Inhibitory Concentration, MIC > 2.0 mg/L). Voriconazole showed good activity against *A. fumigatus* and *A. flavus* strains, except for one clinical strain of *A. fumigatus* whose MIC was 4.0 mg/L. Posaconazole (≤0.25 mg/L) also showed appreciable activity against both species of *Aspergillus*, except for six *A. fumigatus* strains with relatively higher MICs (0.5 mg/L). The MICs for Amphotericin B ranged from 0.06 to 1.0 mg/L for *A. fumigatus*, but were much higher (0.5–8.0 mg/L) for *A. flavus*. Among the echinocandins, caspofungin showed a geometric mean of 0.078 and 0.113 against the clinical and environmental strains of *A. flavus*, respectively, but had elevated minimal effective

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concentrations (MECs) for seven of the *A. fumigatus* strains. Anidulafungin and micafungin exhibited considerable activity against both *A. fumigatus* and *A. flavus* isolates, except for one environmental isolate of *A. fumigatus* that showed an MEC of 1 mg/L to micafungin. Our study proposes that a detailed investigation of the antifungal susceptibility of the genus *Aspergillus* from different regions of Brazil is necessary for establishing a response profile against the different classes of antifungal agents used in the treatment of aspergillosis.

Introduction

Aspergillosis includes a wide range of diseases caused by the filamentous fungus *Aspergillus* that affects mainly the respiratory tract of immunocompromised patients. It is characterized by invasive and chronic pulmonary infection, allergic reaction, or fungal growth. *Aspergillus fumigatus*, followed by *Aspergillus flavus*, are the most common species causing aspergillosis.[1,2] The treatment of these diseases is based on the use of azole antifungal drugs, such as voriconazole (VCZ), which is the treatment of choice, itraconazole (ITZ), posaconazole (PCZ), and more recently, isavuconazole (ISZ).[3,4] Nevertheless, many studies have reported resistance of *A. fumigatus* to the azole antifungal drugs that is often due to the cross-resistance to the agricultural triazoles. Resistance rates vary widely across medical centers around the world, with some studies showing high resistance rates[5–7] and others with rates even lower than 1%.[8,9] As an alternative to the use of azoles, lipid formulations of amphotericin B (AMB) and echinocandins are also being used nowadays in the treatment of aspergillosis.[3,4]

Owing to the widespread use of azoles in the treatment of aspergillosis and the potential consequence of acquiring azole resistance, antifungal susceptibility studies of the concerned fungal species have become increasingly important in order to understand their resistance profile in each medical center and improve local empirical treatment. In this study, we evaluated the susceptibility profile of *A. fumigatus* and *A. flavus*, isolated from patients with different forms of aspergillosis and from the environment in southern Brazil, against azoles, AMB, and echinocandins by using the broth microdilution methodology.

Materials and methods

Aspergillus strains

Twenty-five clinical strains of *A. fumigatus* and 20 of *A. flavus* were isolated from patients with sinusitis, invasive or cutaneous aspergillosis; 20 strains of *A. fumigatus* and 40 of *A. flavus* were isolated from maize crops belonging to the Laboratory of Mycological Research of the Federal University of Santa Maria in southern Brazil. Analyses of macro and micromorphology, growth of *A. fumigatus* at 48 °C, and sequencing of the internal transcribed spacer (ITS 1 and 2) regions were performed to identify the species. ITS region amplifications were performed *via* PCR using ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTAT TGATATG-3′) primers, as described previously.[10] The strains were stored in 10% glycerol at −80 °C and were subcultured on Potato Dextrose Agar (PDA) at 27 °C for three days or until sporulation. In all the tests, *A. fumigatus* ATCC 204305 and *A. flavus* ATCC 204304 strains were used as the internal quality controls.

Antifungal susceptibility testing

The MICs of ITZ, PCZ, VCZ (Sigma–Aldrich, São Paulo, Brazil), and AMB (Leadiant Biosciences, Maryland, USA) and the MECs of the echinocandins such as anidulafungin (AFG; Pharmacia & Upjohn Co. Kalamazoo, MI, USA), caspofungin (CAS; Laboratories Merck Sharp & Dohme-Chibret, Clermont-Ferrand, France), and micafungin (MFG; Astellas Pharma Tech Co., Takaoka, Toyama, Japan) were determined following the recommendations of the EUCAST protocol for filamentous fungi (EUCAST, 2008). All antifungal drugs were solubilized in dimethyl sulfoxide (DMSO) and the working solutions were prepared in RPMI 1640 medium with L-glutamine (Sigma–Aldrich, São Paulo, SP, Brazil). The final drug concentrations ranged from 0.063 to 32.0 mg/L for ITZ and VCZ, 0.008 to 4.00 mg/L for PCZ, 0.016 to 8.00 for AMB, and 0.0005 to 1.00 mg/L for the echinocandins.

Conidial suspensions were obtained from sporulated *Aspergillus* cultures and adjusted to contain $2–5 \times 10^6$ conidia/mL by counting in a hemocytometer. To obtain a final concentration of $2–5 \times 10^5$ conidia/mL, 1:10 dilutions were prepared in sterile distilled water. For the microplate preparation, in each well, 100 μL of the final conidial suspension were added to 100 μL of each of the antifungal drug concentrations. Growth and negative control are included in all tests. The microplates were incubated at 35 °C. The MEC readings for echinocandins, defined as the lowest concentration leading to the growth of the abnormal, branched, and short hyphae as compared to those forming long and unbranched hyphae in the growth control, were performed 24 h post-incubation. The MIC readings for azoles and AMB, defined as the lowest concentration that completely inhibits the growth compared to that obtained in case of the control, were taken 48 h post-incubation.

Data analyses

Geometric mean (GM), MIC/MEC_{50} (minimal inhibitory/effective concentration that inhibits the growth of 50% of the strains) and MIC/MEC_{90} (minimal inhibitory/effective concentration that inhibits the growth