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Evaluation of disk diffusion method compared to broth microdilution for antifungal susceptibility testing of 3 echinocandins against *Aspergillus* spp.☆,☆☆

Antonia Isabel Martos[a,*], Estrella Martín-Mazuelos[a], Ana Romero[a], Carmen Serrano[a], Trinidad González[a], Carmen Almeida[b], Beatriz Puche[a], Emilia Cantón[c], Javier Pemán[c], Ana Espinel-Ingroff[d]

[a] *Microbiology Service, Valme University Hospital, Seville, Spain*
[b] *Biostatistics Service, Research Unity, Valme University Hospital, Seville, Spain*
[c] *Microbiology Service, La Fé University Hospital, Valencia, Spain*
[d] *VCU Medical Center, Richmond, VA, USA*

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ABSTRACT

We tested the susceptibility of caspofungin, micafungin, and anidulafungin against *Aspergillus* spp. isolates by the new Clinical and Laboratory Standards Institute M51-A disk diffusion (DD) and the broth microdilution methods. A total of 65 clinical isolates of *Aspergillus* spp. were evaluated. The DD assay was performed on nonsupplemented Müeller–Hinton agar using caspofungin 2-μg, micafungin 1-μg, and anidulafungin 2-μg disks. Echinocandin minimal effective concentrations (MECs) and inhibition zones (IZs) were read after 24 to 48 (*A. terreus*) h at 35 °C. Caspofungin MECs for all *Aspergillus* spp. strains tested were ≤0.25 μg/mL; IZs were ≥15 mm for most species except for *A. terreus* (11–22 mm). Both micafungin and anidulafungin MECs were ≤0.015 μg/mL, but micafungin IZs were ≥14 mm while anidulafungin IZs were ≥22 mm. As for caspofungin, the DD method could be a useful method for susceptibility testing of micafungin and anidulafungin against *Aspergillus* spp.

1. Introduction

Invasive fungal infections caused by *Aspergillus* spp. have increased in recent years, and they cause significant high morbidity and mortality in the immunocompromised host (De Pauw et al., 2008; Lin et al., 2001; Marr et al., 2002; Metcalf and Dockrell, 2007; Walsh et al., 2008). Appropriate and early therapeutic intervention is critical for managing these infections. The echinocandins are an exciting development in systemic antifungal therapy (Cappelletty and Eiselstein-McKitrick, 2007; Martín-Mazuelos, 2003; Morris and Vilmann, 2006). The principal mechanism of action of echinocandins is the noncompetitive inhibition of (1,3)-β-D-glucan synthase, an essential and exclusive enzyme for the production of cell walls in most fungal species. These antifungal agents exhibit species-dependent fungicidal activity against some *Candida* species, including triazole-resistant isolates (Martín-Mazuelos, 2003; Morrison, 2006; Pfaller et al., 2008), and have good activity against *Aspergillus* spp. (Diekema et al., 2003; Oakley et al., 1998; Tawara et al., 2000).

The Clinical and Laboratory Standards Institute (CLSI) has developed reproducible procedures for testing the susceptibility of filamentous fungi (molds) by a broth microdilution method (CLSI, 2008a) and, more recently, a disk diffusion method (CLSI, 2010) for testing nondermatophyte molds with various antifungal agents (López-Oviedo et al., 2006; Serrano et al., 2004) including caspofungin (Espinel-Ingroff et al., 2007). This latter methodology has not been evaluated for the other 2 echinocandins (anidulafungin and micafungin). The present study was undertaken to evaluate the new disk diffusion (DD) method for testing *Aspergillus* spp. with micafungin, anidulafungin, as well as caspofungin, using nonsupplemented Müeller–Hinton agar (MHA) as previously described by Espinel-Ingroff et al. (2007). DD zone diameters were compared to minimal effective concentrations (MECs) obtained by the reference broth microdilution (MD) method (CLSI, 2008a).

2. Materials and methods

A total of 65 *Aspergillus* isolates (28 *A. fumigatus*, 18 *A. flavus*, 16 *A. terreus*, 2 *A. niger*, and 1 *A. glaucus*) and 4 *Fusarium* spp. isolates were recovered from clinical specimens received at Valme University Hospital (Seville, Spain) and La Fé University Hospital (Valencia, Spain). Identification of each strain was performed using conventional mycological techniques (Murray et al., 2007). Mold isolates were kept in sterile water and subcultured on potato dextrose agar

☆ Performed at the Microbiology Department, Valme University Hospital, Seville-Cádiz Road, 41014 Seville, Spain.

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* Corresponding author. Tel.: +7-955015482; fax: +7-955015487.
E-mail address: aisabel_martos@hotmail.com (A.I. Martos).

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Table 1
In vitro susceptibility results by disk diffusion and broth microdilution methods of caspofungin (CFG) (2 μg/disk), micafungin (MFG) (1 μg/disk), and anidulafungin (AFG) (2 μg/disk) against 69 fungal isolates.

Species (no. of isolates)	IZ-2 (mm)		MEC (μg/mL)	
	Range	AM	Range	GM
A. fumigatus (28)				
CFG	16–29	21.2	≤0.015–0.25	0.06
MFG	14–34	20.1	≤0.015	≤0.015
AFG	24–45	34.5	≤0.015	≤0.015
A. flavus (28)				
CFG	15–26	20.8	≤0.015–0.25	0.06
MFG	17–22	19.7	≤0.015	≤0.015
AFG	22–38	32.3	≤0.015	≤0.015
A. terreus (28)				
CFG	14–24 *(11–22)	18.9 *(16.2)	≤0.015–0.125	0.07
MFG	16–40 *(16–34)	25.5 *(23.9)	≤0.015	≤0.015
AFG	24–36 *(24–35)	31.4 *(30)	≤0.015	≤0.015
A. niger (2)				
CFG	19–20	19.5	0.06	ND
MFG	18–30	23.9	≤0.015	ND
AFG	26–32	29	≤0.015	ND
A. glaucus (1)				
CFG	25	ND	0.125	ND
MFG	15	ND	≤0.015	ND
AFG	35	ND	≤0.015	ND
Fusarium spp. (4)				
CFG	NZD	NZD	>16	ND
MFG	NZD	NZD	>16	ND
AFG	NZD	NZD	>16	ND

IZ = Inhibition zones at the point of marked decrease in fungal density at 24 h; *() = IZ at 48 h; MEC = minimal effective concentrations; AM = arithmetic mean; GM = geometric mean; ND = not determined; NZD = no inhibition zone.

(Becton Dickinson and Company, Bergen, NJ, USA) to ensure viability and purity.

Microdilution method was performed according to the CLSI (2008a) guidelines. Stock inoculum suspensions were prepared from 7-day-old cultures grown on potato dextrose agar and adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.13 (80–82% transmittance) (CLSI, 2008a). Final diluted inoculum sizes for the MD ranged from 0.4×10^4 to 5×10^4 CFU/mL for all the isolates tested as demonstrated by a quantitative colony count on Sabouraud dextrose agar (CLSI, 2008a).

Caspofungin (Merck Research Laboratories, Rahway, NJ, USA), micafungin (Astellas Pharma, Deerfield, IL, USA), and anidulafungin (Pfizer Central Research, New York, NY, USA) were provided as standard powders. Stock drug suspensions were prepared in sterile distilled water (caspofungin and micafungin) and dimethyl sulfoxide (anidulafungin) to yield the final 2-fold drug concentrations of 0.015 to 8 μg/mL. On the day of the test, microdilution trays containing the diluted 2-fold drug concentrations in standard RPMI-1640 medium were inoculated with 100 μL of the diluted 2-fold inoculum and trays were incubated at 35 °C. MECs were determined after 24 to 48 h (*A. terreus* and *Fusarium* spp.). MECs were the lowest drug concentrations that produced small, rounded, compact colonies as compared to the hyphal growth observed in the growth control well (CLSI, 2008a).

The DD method was carried out using caspofungin, micafungin, and anidulafungin disks that were prepared in-house (Arikan et al., 2002, 2003; Maida et al., 2008). Briefly, blank paper disks (Becton Dickinson) were impregnated with 20 μL each of either anidulafungin (100 μg/mL in 1% dimethyl sulfoxide and 0.1% polysorbate 80), caspofungin (100 μg/mL in distilled water), or micafungin (50 μg/mL in distilled water) suspensions. The final drug concentrations in the disks were 2 μg/disk (anidulafungin and caspofungin) and 1 μg/disk (micafungin) (Arikan et al., 2002, 2003; Jones et al., 2007). According to results obtained by Espinel-Ingroff et al. (2007) and as described in the M51-A document, the DD method was performed using nonsupplemented MHA (Becton Dickinson). The entire surface of nonsupplemented MHA plates (90 mm) was inoculated in 3 directions with the undiluted inoculum (equivalent to a 0.5 McFarland turbidity standard or ~10^6 CFU/mL). The inoculated agar was allowed to dry for 15 to 30 min before the disks were applied, and the plates were incubated at 35 °C. The inhibition zone diameters (IZs, in millimeters) were determined at the point of marked decrease in fungal density, ignoring the microcolonies growing inside the zone of inhibition after 24 to 48 h (*A. terreus* and *Fusarium* spp.).

Two quality control (QC) isolates—*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258—and 2 reference strains—*A. fumigatus* ATCC 204305 and *A. flavus* ATCC 204304—were included as controls each time that isolates were tested by both methods and each echinocandin. In addition, 4 well-documented *Fusarium* spp. strains with high echinocandin MECs as in-house controls were evaluated. Stock inoculum suspensions for the QC yeast isolates were prepared following CLSI guidelines for yeast testing (CLSI, 2008b).

The comparative evaluation of the DD and MD methods was performed by calculating ranges and both arithmetic (AM) and geometric (GM) means of both IZs and MEC values. For the correlation between MECs and DD results, a linear regression using the least-square method (Pearson's correlation coefficient; MS Excel software, Microsoft, Redmond, WA, USA) was performed by plotting IZ diameters (in millimeters) against their respective MEC end points of each echinocandin and weighting IZ results according to the different MEC groups.

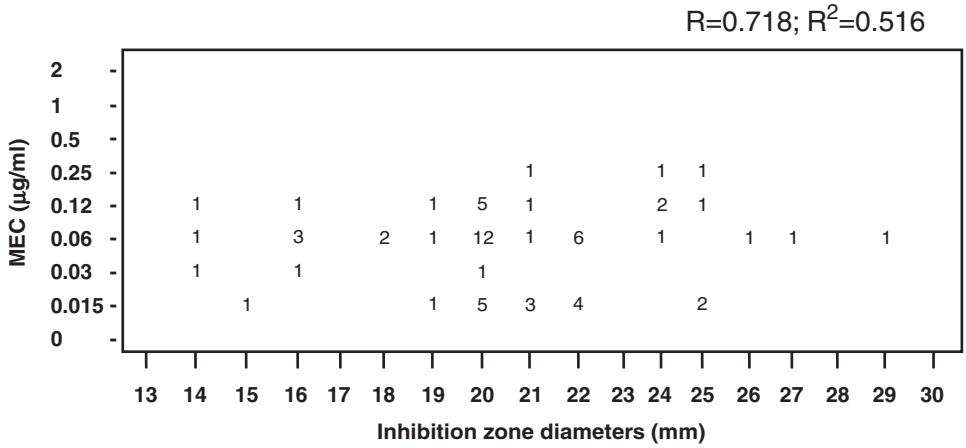

Fig. 1. Scattergram comparing caspofungin disk diffusion zones and broth microdilution reference MECs.