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Feasibility Studies for Anidulafungin Disk Diffusion Susceptibility Testing of Yeast by CLSI M-44 Method

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ABSTRACT

Background: A disk diffusion (DD) method has been successfully developed by CLSI (M-44) to susceptibility (S) test selected azole compounds (fluconazole [FLUC], voriconazole [VORI]) with excellent correlation (r) to MIC values derived from the M-27 broth microdilution method. Anidulafungin, a new echinocandin, DD development was assessed using preliminary modifications by Odabasi et al. (2003) for *Candida* spp.

Methods: Seventy-five (75) *Candida* spp. isolates were tested as follows: *C. albicans* (35), *C. glabrata* (10), *C. parapsilosis* (CPAR; 10), *C. tropicalis* (10), and *C. krusei* (10). Candidate anidulafungin disk concentrations were 1-, 2-, 5- and 10-µg with or without DMSO (0.05-1%) and with or without Tween-80 (0.1%). CLSI method (M-27, M-44) results were compared by regression analysis and 3 recommended quality control strains (QC) were used; all QC results were within published ranges for FLUC and VORI. FLUC, VORI and anidulafungin powder, and FLUC and VORI disks were provided by Pfizer, Inc.

Results: Preliminary studies revealed that neither DMSO (0.05-1%) nor Tween-80 (0.002-2%) alone produced inhibitory zones, however, combined with anidulafungin (1% and 0.1%, respectively) enhanced zones (+13 to 17 mm) especially for CPAR (>15 mm; MICs, 0.5-4 µg/ml). Anidulafungin 1- and 2-µg disks were optimal, with rare zones of >35 mm and acceptable r values (0.84-0.85) for all yeast strains.

Table. Anidulafungin results with four DD concentrations and reference MICs.

Disk Content	Zone range (mm) at 24 hr: CPAR	Zone range (mm) at 24 hr: All Yeast	MIC (µg/ml) range at 24 hr: CPAR	MIC (µg/ml) range at 24 hr: All Yeast	r value:
1-µg	14-21	25-37	0.5-4[a]	≤0.008-0.25[a]	0.84
2-µg	17-23	27-36	-	-	0.85
5-µg	19-26	29-36	-	-	0.86
10-µg	22-31	34-46	-	-	0.78

a. All MIC results were reference tested (M27) and were compared to results from each disk content.

Conclusions: The addition of 1% DMSO and 0.1% Tween-80 in 1- or 2-µg anidulafungin disks optimized the zone diameters for all important yeast species (also CPAR with elevated anidulafungin MIC results) with the 2-µg disk selected for further development. Multicenter studies to establish MIC and DD QC ranges, and larger isolate volume regression studies are now warranted.

INTRODUCTION

Fungal infections caused by *Candida* spp. may result in significant morbidity and mortality, prolonged hospitalization, and increased health care costs. Early diagnosis and the initiation of appropriate antifungal therapy are fundamental for influencing positive patient outcomes. The Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards [NCCLS]) broth microdilution M27-A2 (2002) method has emerged as the reference standard procedure for determining MIC susceptibilities to the wide spectrum of licensed antifungal agents. This method requires a greater level of time, preparation, and training that could be significant for laboratories having low antifungal testing volume and for high-volume testing laboratories that must budget a greater staff commitment for antifungal test performance and interpretation of results.

A relatively rapid and technically more simple disk diffusion method has been successfully developed by CLSI (M44-A; 2004) that demonstrated excellent correlation for selected azole compounds (fluconazole and voriconazole) when compared to MIC values derived from the M27-A2 method. This agar-based method offers the potential benefits of reduced preparation time, decreased time to reporting (24 vs 48h), improved ease of use, and minimal training. Expansion of this method to other licensed antifungal agents or classes should be of great interest to clinical laboratories worldwide.

Anidulafungin, a new echinocandin, is one such agent which has demonstrated activity against a wide variety of commonly isolated *Candida* spp. including those species with reduced susceptibilities to some azoles. To determine the feasibility of using the disk diffusion method against common *Candida* spp., anidulafungin disks were prepared using specific solvents (water, DMSO) and a defined surfactant (polysorbate-80) alone and in combination as previously described by Odabasi et al. (2003).

MATERIALS AND METHODS

Fungal Isolates: The organism collection included a total of 75 recent clinical isolates of *C. albicans* (n = 35); *C. glabrata* (n = 10); *C. parapsilosis* (n = 10); *C. tropicalis* (n = 10); and *C. krusei* (n = 10), some *C. krusei*, and *C. glabrata* strains with decreased susceptibilities to azoles. In addition, the quality control (QC) strains *C. krusei* ATCC 6258, *C. parapsilosis* ATCC 22019 and *C. albicans* ATCC 90028 (disk diffusion only) were tested concurrently with each batch of organisms. Prior to processing, all isolates were subcultured from room temperature water storage onto potato dextrose agar (Remel, Inc., Lenexa, KS) to ensure viability and optimal growth.

Susceptibility testing: Anidulafungin reagent grade powder and commercially available fluconazole (25-µg) and voriconazole (1-µg) disks were provided by the sponsor (Pfizer, Inc., New York, NY). Paper disks containing a final concentration of 1-, 2-, 5-, 10- and 20-µg of anidulafungin were produced using DMSO and polysorbate-80 as described by Odabasi et al., 2003.

Antifungal broth microdilution susceptibility testing was performed as described by M27-A2 CLSI methods (2002) for anidulafungin and comparator agents. Concurrently, disk diffusion testing was performed by applying an inoculation equivalent to a 0.5 McFarland turbidity standard to the surface of a 150 mm Mueller-Hinton agar plate supplemented with 2% glucose and 0.5 µg/ml methylene blue (Hardy Diagnostics, Santa Maria, CA) using a sterile cotton swab applicator as described in the M44-A CLSI standard (2004).

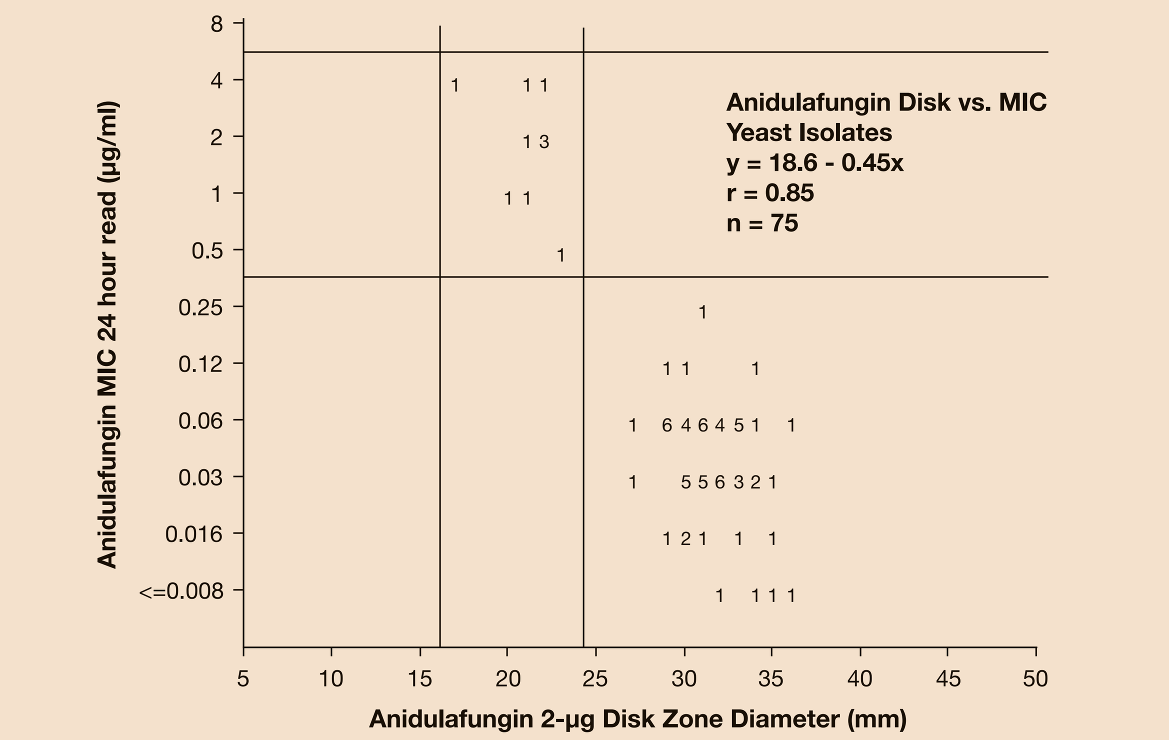
RESULTS

- Initial tests using three QC strains and disks containing DMSO or methanol and five log_{10} concentrations of polysorbate-80 (0.0002, 0.002, 0.02, 0.2, 2%) demonstrated there was no inhibitory effects (no zones of inhibition) for the solvents or the surfactant alone.
- 1-µg anidulafungin disks were produced with two concentrations of DMSO (0.05 or 1%) with or without 0.1% polysorbate-80. The addition of the polysorbate-80 increased zone diameter ranges for the replicate testing of three QC strains from 6-14 mm to 19-34 mm (lowest for *C. parapsilosis* ATCC 22019; range 19-22 mm).
- Further testing of the 20-µg anidulafungin disk was discontinued because early tests showed excessively large zone diameters (>50 mm).
- Table 1 shows the results for eight clinical isolates from four *Candida* species demonstrating the influences of varying DMSO and polysorbate-80 combinations with the 1-µg anidulafungin disk concentration. DMSO concentration effects on zone diameters remained insignificant, but the 0.1% polysorbate-80 component enlarged the zones of inhibition by an average of 14.8 mm.
- Figure 1 shows the scattergram comparing anidulafungin MIC results (24-hour reading) and zone diameters around the proposed 2-µg disk (with 1% DMSO and 0.1% polysorbate-80) for the 75 tested *Candida* spp. strains (r = 0.85). Scattergrams with the 5- and 10-µg anidulafungin disks had zones that were very large at 19-36 mm (median, 32 mm) and 29-49 mm (median, 39 mm), respectively.
- The anidulafungin 1-µg disks also performed well, but produced a few *C. parapsilosis* zones at <20 mm and one zone at only 14 mm, favoring the 2-µg disk results that were slightly larger and created excellent separation between *C. parapsilosis* strains (17-23 mm) and those zones for other *Candida* spp. isolates (four species; 27-34 mm).
- Fluconazole and voriconazole tested as disk diffusion and MIC control agents had acceptable intermethod categorical agreement of 85.3% (fluconazole) and 90.7% (voriconazole), without false-susceptible (very major) errors.

Table 1. Testing results for eight wild type yeast isolates using 1-µg anidulafungin disks produced with various solvent concentrations in water, and with or without polysorbate-80 (P-80; zone diameters in mm).

Organism (collection no.)	Anidulafungin alone: 0.05% DMSO	Anidulafungin alone: 1% DMSO	Anidulafungin with 0.1% P-80: 0.05% DMSO	Anidulafungin with 0.1% P-80: 1% DMSO
C. parapsilosis				
30-13859A	6	6	18	18
21-1939A	6	6	17	18
C. albicans				
105-2081A	14	14	30	30
105-2902A	14	14	31	32
103-4967A	13	13	29	30
C. krusei				
27-12971A	11	11	25	25
17-1359A	12	12	26	26
C. tropicalis				
03-8792A	13	13	28	27

Figure 1. Scattergram comparing anidulafungin MIC results (read at 24 hours) to zone diameters around a 2-µg anidulafungin disk interpreted after 24 hours incubation (M44-A method; 75 strains of *Candida* spp.). Vertical and horizontal lines are those tentative interpretive criteria defining a *C. parapsilosis* population (MIC, 0.5-4 µg/ml; zones 17-24 mm) and the more susceptible other *Candida* spp. (MIC, ≤0.25 µg/ml; zones at ≥25 mm).



CONCLUSIONS

- Using procedural details described by Odabasi et al. (2003) for anidulafungin disk susceptibility testing of *C. parapsilosis*, we validated the 2-µg anidulafungin disk content against a more comprehensive collection of yeast species.
- Multicenter studies to establish anidulafungin MIC and disk diffusion QC ranges using CLSI broth microdilution and disk diffusion methods are now warranted.
- MIC versus disk diffusion zone diameter regression studies with a larger collection of clinical yeast isolates (≥300 strains) are also necessary to complete anidulafungin in vitro test development.

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