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Antifungal Susceptibility Patterns of a Global Collection of Fungal Isolates and Polysorbate-80 Effect on the Susceptibility of the Antifungal Classes

M CASTANHEIRA, SA MESSER, RR DIETRICH, PR RHOMBERG, RN JONES, MA PFALLER
JMI Laboratories, North Liberty, Iowa, USA

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JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
ph. 319.665.3370, fax 319.665.3371
mariana-castanheira@jmilabs.com

AMENDED ABSTRACT

Background: The importance of antifungal surveillance was highlighted by the increasing resistance among certain species and breakthrough infections. We evaluated 1,846 fungal clinical isolates against 9 antifungals using CLSI reference broth microdilution methods (BMD). Additionally, 1,202 isolates were tested using polysorbate-80 (P-80).

Methods: 1,846 isolates collected in 2013 (31 countries) were tested by CLSI BMD and interpretive criteria. Echinocandins (EC), amphotericin B (AMB) and fluconazole (FLC) were also tested using 0.002% P-80 supplemented broth. Isolates were identified using MALDI-TOF MS and/or DNA sequencing.

Results: EC, AMB and FLC were active against common *Candida* spp. (**Table**). EC-resistance ranged from 0.0 to 2.8% (anidulafungin for *C. glabrata* [CGLA]). 11.9 and 11.6% of the CGLA and *C. tropicalis* were resistant to FLC, respectively. Two *A. fumigatus* displayed elevated MIC values for itraconazole (≥4 µg/ml). All *C. neoformans* had MIC < epidemiological cutoff values for azoles. P-80 lowered the MIC values for EC for all species, but not for FLC. AMB MIC values were lower and ranges broader (0.03-0.5 µg/ml) when compared with reference BMD (0.5-2 µg/ml).

Conclusions: EC and azoles were potent against yeasts and moulds. P-80 use broadened MIC ranges for AMB; however, differences in the growth patterns in RPMI + P-80, requirement for new QC ranges and a possible effect in cell growth reported previously in bacteria might be an impediment to the use of P-80 for antifungal BMD testing.

Organism (no. tested [no. tested with P-80])	MIC/MEC$_{50/90}$ for CLSI BMD (with P-80)			
	Anidulafungin	Caspofungin	Amphotericin B	Fluconazole
C. albicans (712 [475])	0.015/0.06 (≤0.008/≤0.008)	0.03/0.03 (≤0.008/≤0.008)	1/1 (0.06/0.12)	0.12/0.25 (0.25/0.25)
C. glabrata (251 [156])	0.06/0.12 (0.015/0.015)	0.03/0.06 (≤0.008/≤0.008)	1/1 (0.12/0.12)	8/64 (4/32)
C. parapsilosis (215 [149])	2/2 (1/2)	0.25/0.5 (0.06/0.06)	1/1 (0.12/0.25)	1/2 (1/4)
C. tropicalis (155 [90])	0.015/0.03 (≤0.008/≤0.008)	0.03/0.03 (≤0.008/≤0.008)	1/1 (0.06/0.12)	0.5/32 (0.5/1)
C. krusei (49 [29])	0.06/0.06 (0.03/0.03)	0.12/0.25 (0.03/0.03)	1/2 (0.25/0.25)	32/64 (32/64)
A. fumigatus (142 [94])	≤0.008/0.03 (≤0.008/≤0.008)	0.03/0.03 (≤0.008/≤0.008)	2/2 (0.25/0.5)	-/-

INTRODUCTION

Invasive fungal infections constitute an ever-increasing cause of morbidity and mortality among immunocompromised individuals, those who have undergone intra-abdominal surgery, neonates, and the elderly. The most frequent infections reported are invasive candidiasis, other mycoses (e.g. pseudallescheriasis, fusariosis and other unspecified mycoses), and invasive aspergillosis. Concurrent with increasing numbers of invasive fungal infections, surveillance programs have become important in defining the species distribution and antifungal resistance profiles of the responsible pathogens; and thus providing information necessary for appropriate empiric antifungal therapy.

In this study, we examined the activity of systemic antifungal agents tested against 1,846 contemporary clinical fungal strains collected from sterile body sites, respiratory tract and bloodstream infections worldwide during 2013. Additionally, we screened the echinocandin non-susceptible *Candida* spp. isolates for *fks* mutations and tested a large subset of isolates (n=1,202) in the presence of the surfactant polysorbate-80 that has been used in antibacterial susceptibility testing to reduce compound loss in frozen-form plate preparation and increase the separation among susceptible and resistant isolates.

MATERIALS AND METHODS

Organisms. A total of 1,846 clinical isolates were collected as part of a global surveillance program and included: 1,514 (82.0%) *Candida* spp., 114 (6.2%) non-candidal yeasts, including 84 (4.5%) *C. neoformans*, 196 (10.6%) *Aspergillus* spp., and 22 (1.2%) other moulds. Isolates were geographically distributed among Europe (41.0%), Asia-Pacific (24.5%), North America (23.5%) and Latin America (11.0%). In each case, collection was approved by the appropriate institutional review board. Each participating center recovered consecutive non-duplicate isolates from patients with bloodstream infections (1,077 strains), normally sterile body fluids, tissues and abscesses (204 strains), respiratory tract specimens (226 strains) and other unknown specimens (339 strains). Isolates were submitted to JMI Laboratories (North Liberty, Iowa, USA), where the ID was confirmed by morphological, biochemical, and molecular methods as described elsewhere.

Antifungal susceptibility testing. All yeast isolates were tested for in vitro susceptibility to the echinocandins (anidulafungin, caspofungin, micafungin) and the triazoles (fluconazole, itraconazole, posaconazole, voriconazole) using CLSI BMD according to the M27-A3 document. MIC results for all agents were read following 24-h of incubation when tested against *Candida* spp., whereas MIC endpoints for the triazoles were read after 48-h when tested against non-*Candida* yeasts. In vitro susceptibility testing of *Aspergillus* spp. and other moulds against the echinocandins and triazoles (itraconazole, posaconazole, voriconazole) was performed by BMD as described in CLSI document M38-A2. The triazole MICs and echinocandin minimum effective concentrations (MEC) were determined as described in the CLSI reference method.

A total of 1,202 isolates randomly selected were tested for the echinocandins, amphotericin B and fluconazole in the presence of polysorbate-80 (P-80; Sigma-Aldrich, St. Louis, Missouri, USA). P-80 was used at panel preparation by adding 0.002% to RPMI broth used to dilute antimicrobial solutions.

CLSI clinical breakpoint (CBP) values (CLSI M27-S4 guideline) were used for the most common species and epidemiologic cutoff values (ECVs) were applied for less common species of *Candida*, non-*Candida* yeasts, *Aspergillus* spp., or the non-*Aspergillus* moulds. Quality control was performed as recommended in CLSI documents M27-A3 and M38-A2 using *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019.

Sequencing of the *fks* HS regions. *Candida* spp. isolates with MIC values higher than the ECV had the hotspots of the genes encoding the 1,3 β-D-glucan synthase subunits sequenced. DNA extraction was performed using the Qiagen QIAamp DNA mini kit (Qiagen, Hilden, Germany) in the QiaCube automated platform (Qiagen). PCR amplification was carried out using previously described oligonucleotides for the *fks1* and *fks2* HSs 1 and 2. Amplicons were sequenced on both strands and the nucleotide sequences and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, WI).

RESULTS

- Echinocandins had comparable activity when tested against *C. albicans* isolates (MIC$_{50/90}$, 0.015/0.06, 0.03/0.03 and 0.015/0.03 µg/ml for anidulafungin, caspofungin and micafungin, respectively; **Table 1**). Fluconazole and voriconazole inhibited 99.6% and 99.7%, respectively, of the *C. albicans* at current breakpoints (**Table 1**).
- Fluconazole resistance was noted among 12.0% of *C. glabrata*, and these strains were noted in 10 countries and a total of 10 out of 30 isolates were detected in USA hospitals. Eleven *C. glabrata* strains displayed elevated echinocandin MIC values (>ECV) and mutations were detected in three isolates and were all in the *fks2* HS1 (F659 deletion, D666E/K753Q and S663P; **Table 2**).
- Susceptibility against anidulafungin among *C. parapsilosis* was 95.3% and 100.0% for micafungin and caspofungin. Fluconazole and voriconazole inhibited 94.0 and 99.1% of the isolates, respectively, at clinical breakpoint values (**Table 1**).
- Eighteen azole non-susceptible *C. tropicalis* strains were noted from the Asia-Pacific Region: China (n=7), Thailand (n=5), Australia and Singapore (one each). The remaining four isolates were from the USA (n=2; two hospitals) or Czech Republic (n=2; one hospital). Among four isolates displaying elevated echinocandin MIC values, two resistant according to clinical breakpoints displayed mutations on F641(L/S) (**Table 2**).
- All azoles tested displayed 100.0% activity against *C. neoformans* and MIC$_{50/90}$ values were 2/4, 0.03/0.06 and 0.12/0.25 µg/ml for fluconazole, voriconazole and posaconazole, respectively (**Table 1**).
- Echinocandins displayed good activity against *A. fumigatus* (MEC$_{50}$, ≤0.008, ≤0.008 and 0.03 µg/ml for anidulafungin, micafungin and caspofungin, respectively; **Table 1**). Two isolates had MIC values above the ECV established for voriconazole and were considered non-wildtype. These isolates are currently being further investigated.
- MIC results with P-80 were usually lower for the echinocandin compounds for all species and a comparison for the four most common species tested is displayed in **Figure 1**. Similar differences were not observed with fluconazole and results changed only ±1 dilution.
- Amphotericin B results ranged mostly from 0.25 to 2 µg/ml and displayed a broader and lower range when tested in the presence of P-80 (≤0.015 to 0.5 µg/ml for most results).

Table 2. Summary of FKS alterations detected in *Candida* spp. strains as part of the 2013 international surveillance program.

Organism	State and/or Country	CLSI method (µg/ml):			1,3-β-D-glucan synthase mutations:[a]			
		Anidulafungin	Caspofungin	Micafungin	*fks1* HS1	*fks1* HS2	*fks2* HS1	*fks2* HS2
C. glabrata	Canada	1	0.5	0.5	WT	WT	F659 deletion	WT
C. glabrata	Germany	0.25	0.12	0.03	WT	WT	WT	WT
C. glabrata	Germany	0.5	0.5	0.06	WT	WT	D666E, K753Q	WT
C. glabrata	Germany	0.5	0.12	0.03	WT	WT	WT	WT
C. glabrata	Turkey	0.5	0.5	0.06	WT	WT	WT	WT
C. glabrata	Brazil	0.25	0.06	0.03	WT	WT	WT	WT
C. glabrata	France	2	1	0.5	WT	WT	S663P	WT
C. glabrata	NY, USA	1	1	0.12	WT	WT	WT	WT
C. glabrata	NY, USA	0.25	0.12	0.03	WT	WT	WT	WT
C. glabrata	Australia	0.12	0.06	0.06	WT	WT	WT	WT
C. glabrata	Australia	0.25	0.06	0.015	WT	WT	WT	WT
C. tropicalis	IN, USA	1	0.5	0.5	F641L	WT	NT	NT
C. tropicalis	CA, USA	2	2	1	F641S	WT	NT	NT
C. tropicalis	Spain	0.06	0.06	0.12	WT	WT	NT	NT
C. tropicalis	Czech Republic	0.03	0.03	0.12	WT	WT	NT	NT
C. pelliculosa[b]	China	0.12	0.25	0.25	WT	WT	NT	NT

a. NT= not tested, WT= wildtype/no mutations detected.
b. Results were compared to those of a susceptible *C. pelliculosa* isolate from the same hospital.

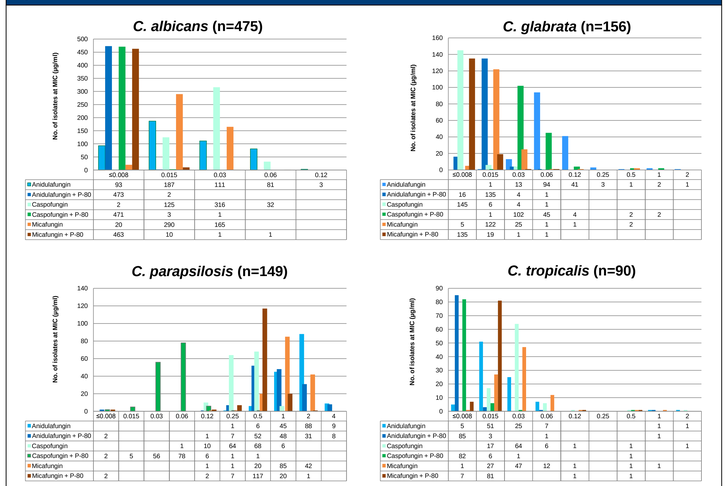
Table 1. Activity of antifungal agents tested against most common fungal pathogens collected in a global surveillance study during 2013.

Organism (no. tested) Antifungal agent	MIC/MEC$_{50}$ (µg/ml)	MIC/MEC$_{90}$ (µg/ml)	Range (µg/ml)	CLSI[a] %S[a] / %R[a]	ECV[b] %WT[b] / %NWT[b]
C. albicans (712)					
Anidulafungin	0.015	0.06	≤0.008 – 0.12	100.0 / 0.0	100.0 / 0.0
Caspofungin	0.03	0.03	≤0.008 – 0.06	100.0 / 0.0	100.0 / 0.0
Micafungin	0.015	0.03	≤0.008 – 0.03	100.0 / 0.0	100.0 / 0.0
Fluconazole	0.12	0.25	≤0.06 – >128	99.6 / 0.4	98.5 / 1.5
Voriconazole	≤0.008	0.015	≤0.008 – >8	99.7 / 0.3	99.2 / 0.8
Posaconazole	0.06	0.06	≤0.008 – >8	- / -	95.1 / 4.9
C. glabrata (251)					
Anidulafungin	0.06	0.12	0.015 – 2	96.0 / 2.4	96.0 / 4.0
Caspofungin	0.03	0.06	0.015 – 1	98.0 / 2.0	98.0 / 2.0
Micafungin	0.015	0.03	≤0.008 – 0.5	98.8 / 0.8	97.6 / 2.4
Fluconazole	8	64	0.12 – >128	- / 12.0	79.3 / 20.7
Voriconazole	0.12	1	≤0.008 – 8	- / -	88.0 / 12.0
Posaconazole	1	2	0.06 – >8	- / -	95.6 / 4.4
C. parapsilosis (215)					
Anidulafungin	2	2	0.25 – 4	95.3 / 0.0	100.0 / 0.0
Caspofungin	0.25	0.5	0.06 – 1	100.0 / 0.0	100.0 / 0.0
Micafungin	1	2	0.12 – 2	100.0 / 0.0	100.0 / 0.0
Fluconazole	1	2	0.25 – 32	94.0 / 2.3	80.9 / 19.1
Voriconazole	0.015	0.06	≤0.008 – 0.5	99.1 / 0.0	99.1 / 0.9
Posaconazole	0.12	0.12	0.03 – 0.5	- / -	98.6 / 1.4
C. tropicalis (155)					
Anidulafungin	0.015	0.03	≤0.008 – 2	98.7 / 1.3	98.7 / 1.3
Caspofungin	0.03	0.03	0.015 – 2	98.7 / 0.6	98.7 / 1.3
Micafungin	0.03	0.06	≤0.008 – 1	98.7 / 0.6	97.4 / 2.6
Fluconazole	0.5	32	0.12 – >128	88.4 / 11.6	87.7 / 12.3
Voriconazole	0.03	1	≤0.008 – >8	88.4 / 10.3	87.7 / 12.3
Posaconazole	0.06	0.25	0.015 – >8	- / -	84.5 / 15.5

Organism (no. tested) Antifungal agent	MIC/MEC$_{50}$ (µg/ml)	MIC/MEC$_{90}$ (µg/ml)	Range (µg/ml)	CLSI[a] %S[a] / %R[a]	ECV[b] %WT[b] / %NWT[b]
C. krusei (49)					
Anidulafungin	0.06	0.06	0.03 – 0.06	100.0 / 0.0	100.0 / 0.0
Caspofungin	0.12	0.25	0.06 – 0.25	100.0 / 0.0	100.0 / 0.0
Micafungin	0.06	0.12	0.06 – 0.12	100.0 / 0.0	100.0 / 0.0
Fluconazole	32	64	8 – 64	- / -	77.6 / 22.4
Voriconazole	0.25	0.25	0.06 – 0.5	100.0 / 0.0	100.0 / 0.0
Posaconazole	0.25	0.5	0.03 – 0.5	- / -	100.0 / 0.0
C. neoformans (84)					
Fluconazole	2	4	0.25 – 8	- / -	100.0 / 0.0
Voriconazole	0.03	0.06	≤0.008 – 0.12	- / -	100.0 / 0.0
Itraconazole	0.12	0.25	0.03 – 0.5	- / -	100.0 / 0.0
Posaconazole	0.12	0.25	0.06 – 0.25	- / -	100.0 / 0.0
Voriconazole	0.25	0.25	0.06 – 0.5	100.0 / 0.0	100.0 / 0.0
A. fumigatus (142)					
Anidulafungin	≤0.008	0.03	≤0.008 – 0.06	- / -	- / -
Caspofungin	0.03	0.03	≤0.008 – 0.06	- / -	100.0 / 0.0
Micafungin	≤0.008	0.03	≤0.008 – 0.03	- / -	- / -
Voriconazole	0.25	0.5	0.12 – 2	- / -	98.6 / 1.4
Itraconazole	1	1	0.25 – 4	- / -	98.6 / 1.4
Posaconazole	0.25	0.5	0.06 – 1	- / -	98.6 / 1.4
Amphotericin B	2	2	0.25 – 2	- / -	100.0 / 0.0

a. Clinical and Laboratory Standards Institute (CLSI), susceptible (S), resistant (R).
b. Epidemiologic cutoff value (ECV), wild-type (WT), non wild-type (NWT).

Figure 1. Comparison of echinocandin MIC distributions with and without P-80 for the four most prevalent species tested.



CONCLUSIONS

- The echinocandins remain very active against contemporary isolates of *Candida* species. Among 16 echinocandin non-wildtype isolates, only five had *fks* HS mutations (three *C. glabrata* and two *C. tropicalis*) and isolates with the absence of mutations usually displayed lower MIC values for the echinocandins.
- In general, the use of P-80 seemed to improve the separation of resistant and susceptible isolates for the echinocandins and for the testing of amphotericin B increasing the range of MIC values obtained. No effects were observed with fluconazole. However, overall the growth in the plates were considerably different in the presence of P-80 (button-like instead a flocculent suspension) and retraining of clinical mycologists might be required if this surfactant is added to antifungal susceptibility testing.
- Surveillance of antifungal agents seems to be prudent and although resistance to current agents is still uncommon, breakthrough infections and increasing resistance in certain geographic areas has been noted.

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