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New CLSI Clinical Breakpoints and Epidemiological Cutoff Values Applied to Characterize Resistance in the SENTRY Antifungal Surveillance Program (2010-2011)

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Abstract

Background: The SENTRY Program monitors global susceptibility (S) and resistance (R) rates of newer and established antifungal agents. We report the echinocandin (EC) and triazole (TZ) antifungal S patterns for 3,416 contemporary clinical isolates of yeasts and moulds.

Methods: 3,416 isolates were obtained from 98 laboratories in 34 countries during 2010-2011. Yeasts not presumptively identified (ID) by CHROMagar, trehalose test and growth at 45°C were sequence ID using 1-2 genes, as well as all moulds (2 genes). S testing was performed against 7 antifungals (anidulafungin [ANF], caspofungin [CSF], micafungin [MCF], fluconazole [FLC], itraconazole, posaconazole [PSC], voriconazole [VRC]) using CLSI methods. R rates to all agents were determined using the new CLSI clinical breakpoints (CBP) and epidemiological cutoff values (ECV) criteria, as appropriate. Sequencing of *fks* hot spots was performed for EC non-WT strains.

Results: Isolates included 3,107 *Candida* (20 species), 146 *Aspergillus* (11 species), 84 *C. neoformans* (CN), 40 other moulds (17 species), and 39 other yeasts (7 species). Among *Candida,* R to ANF, CSF, and MCF was low (0.0-1.7%). *C. albicans* (CA) and *C. glabrata* (CG) that were R to ANF, CSF, or MCF were shown to have *fks* mutations. R to FLC was low among isolates of CA (0.4%), *C. tropicalis* (CT; 1.3%) and *C. parapsilosis* (CP; 3.0%); 8.8% of CG were FLC-R. Among EC-R CG isolates from 2011, 38% were FLC-R. VRC was active against all *Candida* spp. except CG (10.5% non-WT) whereas PSC showed decreased activity against CA (7.8% non-WT) and *C. krusei* (15.2% non-WT). All agents except for the ECs and PSC were active vs. CN and the TZs were active vs. other yeasts (MIC_{90}, 2 µg/ml). The ECs and TZs were active vs. *Aspergillus* (MIC/MEC_{90} range, 0.015-2 µg/ml), but were not active vs. other moulds (MIC/MEC_{90} range, 4->16 µg/ml).

Conclusions: Overall, EC and TZ R rates were low; however, FLC and co-R among CG strains warrants continued close surveillance.

Introduction

The frequency of invasive fungal infections (IFI) due to opportunistic fungal pathogens has clearly increased in recent years. The most well-known causes of opportunistic mycoses include *Candida* spp., *Cryptococcus neoformans* and *Aspergillus fumigatus*. Infections due to these organisms are associated with significant morbidity and mortality as well as excess costs. Optimal therapy of these IFIs is complicated by the lack of rapid, accurate diagnostic methods and the emergence of resistance to both azole and echinocandin antifungal agents. These considerations underscore the importance of understanding both the epidemiology and resistance profiles of contemporary isolates of these important pathogens.

Since the debut of fluconazole in 1990, the introduction of yeast- and mould-active triazoles (posaconazole, voriconazole) and echinocandins (anidulafungin, caspofungin, micafungin) has vastly increased treatment options for prophylaxis and empiric therapy of IFIs. Among clinical isolates of fungi, resistance to antifungal agents is relatively uncommon; however, increased resistance has been reported among patients receiving long-term therapy to both established and newer antifungal agents, emphasizing the need for vigilant surveillance, an expanded role for antifungal susceptibility testing, and the use of molecular techniques to enhance our understanding of either known or developing mechanisms of resistance.

In this study, we analyzed 3,416 fungal clinical isolates collected worldwide as part of the SENTRY Antifungal Surveillance Program using molecular techniques for species identification of unusual organisms and reference susceptibility methods for new and established antifungal agents.

Methods

A total of 3,416 fungal strains were collected during 2010 and 2011 from medical centers in North America, Latin America, Europe, and the Asia-Pacific region as part of the SENTRY Program. Each center recovered consecutive, non-duplicate strains from patients with bloodstream infections (2,227 strains), normally sterile body fluids, abscesses, and tissues (425 strains), respiratory tract infections (352 strains) and others/unknown (412 strains). Strains were identified at the participating medical centers and submitted to JMI Laboratories (North Liberty, Iowa, USA), where they were confirmed by morphological methods and biochemical tests. Yeast isolates were subcultured and screened using CHROMagar® *Candida* (Becton Dickinson, Sparks, Maryland, USA) in order to differentiate *Candida albicans*/*dubliniensis*, *C. tropicalis*, and *C. krusei* and ensure purity. Biochemical tests including trehalose assimilation (*C. glabrata*) or growth at 45°C (*C. albicans/C. dubliniensis*) were additionally used to establish identification. Molecular methods were performed on those species of yeasts and moulds that could not be definitively identified using conventional methods or that presented unusual phenotypic or biochemical profiles. Yeasts not identified by morphological or biochemical tests were identified using sequence-based methods for internal transcribed spacer (ITS) region, 28s ribosomal subunit, IGS1 (*Trichosporon* spp.) or IGS (*Debaromyces* spp.). All mould isolates were subcultured and analyzed by ITS followed by specific molecular species identification within genera: β-tubulin for *Aspergillus* spp., translation elongation factor for *Fusarium* spp., 28S for all other genera of moulds. Nucleotide sequences were examined using Lasergene® software (DNA Star, Madison, Wisconsin, USA) and then compared to database sequences using BLAST (http://www.nbcti.nlmnih.gov.blast). *Fusarium* spp. isolates were analyzed for TEF sequences using the *Fusarium*-ID database (http://www.isolate.fusariumdb.org/index.php) and the *Fusarium* multilocus sequence typing (MLST) database (http://chs.knaw.nl/fusarium/).

All isolates were tested by the broth microdilution method as described in the Clinical and Laboratory Standards Institute (CLSI) reference documents M27-A3 (yeasts) and M38-A2 (moulds). Testing was performed against nine antifungal agents using sequential two-fold dilutions in RPMI 1640 broth buffered with MOPS (morpholinepropanesulfonic acid) and 0.2% glucose. Antifungal agents and ranges tested were anidulafungin (0.008-16 µg/ml), caspofungin (0.008-16 µg/ml), micafungin (0.008-16 µg/ml), fluconazole (0.06-128 µg/ml), posaconazole (0.008-8 µg/ml), itraconazole (0.008-8 µg/ml), voriconazole (0.008-8 µg/ml), flucytosine (0.5-32 µg/ml) and amphotericin B (0.12-2 µg/ml). For yeasts, panels were inoculated with a resulting final standardized cell concentration of $0.5\text{-}2.5 \times 10^3$ cells/ml, then read visually following 24 or 48h of incubation at 35°C. For moulds, conidial suspensions were established spectrophotometrically and diluted to a final test concentration of $0.4\text{-}5.0 \times 10^4$ CFU/ml, followed by visual determination of MIC/MEC after 24, 48, or 72 hours of incubation at 35°C. Quality control was performed as recommended in M27-A3 (*Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258) and M38-A2 (*Aspergillus fumigatus* MYA-3626 and *Aspergillus flavus* ATCC 204304). Interpretation of results followed guidelines recently published.

Following susceptibility testing, amplification and sequencing of *fks* hot spots (HS) were performed on those *Candida* spp. displaying resistant echinocandin MIC values.

Results

- Among the 3,416 isolates tested, 3,107 (90.9%) were *Candida* spp., 123 (3.6%) were non-candidal yeasts, including 84 (2.6%) *C. neoformans*, 146 (4.3%) were *Aspergillus* spp., and 40 (1.2%) were other moulds. Isolates were geographically distributed among North America (39.5%), Europe (34.9%), Latin America (14.4%) and the Asia-Pacific region (11.2%).
- Overall, *C. albicans* strains were very susceptible to all compounds tested. Echinocandins (anidulafungin, caspofungin and micafungin) were very active against *C. albicans* (MIC_{50}, 0.03 µg/ml for all three compounds; **Tables 1** and **2**). Fluconazole and voriconazole inhibited >99% of the *C. albicans* at current breakpoints (**Table 1**).
- Among three echinocandin-resistant *C. albicans* strains tested for *fks* hot spot (HS) mutations, *fks1* HS1 mutations S645P (2 strains, Sweden and China) and S649P (1 strain, Scotland; **Table 3**) were detected.
- Echinocandins inhibited 93.7 to 97.9% of the *C. glabrata* using breakpoint criteria (**Table 1**). Fluconazole resistance was noted among 8.8% of the *C. glabrata* strains. Ten of twelve echinocandin-resistant *C. glabrata* displayed mutations on *fks1* HS1 (two strains) and eight had mutations on the *fks2* HS1 (**Table 3**).
- Applying current clinical breakpoints, only 0.0 to 0.5% of the *C. parapsilosis* isolates were categorized as resistant to echinocandin compounds (**Tables 1** and **2**). Fluconazole and voriconazole inhibited 94.7 and 97.2% of the isolates at clinical breakpoint values, respectively. Voriconazole displayed greater activity against these strains (MIC_{50}, 0.015 µg/ml; **Table 1**).
- All *C. tropicalis* were susceptible to echinocandins (**Table 1**). Fluconazole and voriconazole (MIC_{50}, 0.25 and 0.015 µg/ml; respectively) inhibited 96.5 and 96.2% of the strains at current breakpoints (**Table 1**). Additionally, the activity of itraconazole and posaconazole was also very good against these strains (MIC_{50}, 0.06 µg/ml for both compounds; **Table 1**).
- The activity of anidulafungin against *C. krusei* (MIC_{50}, 0.06 µg/ml; **Table 1**) was two-fold greater than the activities of caspofungin and micafungin (MIC_{50}, 0.12 and 0.12 µg/ml, respectively) and only 1.3% of the strains were considered non-WT to anidulafungin and micafungin. Voriconazole was active against 93.7% of the isolates using current breakpoints.
- Fluconazole and other triazoles displayed good activity against *C. neoformans* (**Table 1**) and $MIC_{50/90}$ values were 4/8, 0.03/0.06 and 0.12/0.5 µg/ml for fluconazole, voriconazole and posaconazole, respectively.
- Mould-active triazoles displayed good activity against *A. fumigatus*, and voriconazole and posaconazole displayed similar activity against these strains ($MIC_{50/90}$, 0.5/0.5 and 0.25/0.5 µg/ml; respectively; **Table 1**). Echinocandins exhibited good activity against *A. fumigatus* and anidulafungin (MEC_{50}, 0.015 µg/ml) activity was similar to caspofungin (MEC_{50}, 0.03 µg/ml) but slightly less than micafungin (MEC_{50}, ≤0.008 µg/ml).

Table 1. Activity of antifungal agents against fungal clinical isolates collected as part of the SENTRY Program during 2010 and 2011.

Organism (no.tested)/ antimicrobial agent	MIC/MEC (µg/ml) 50%	90%	Range	CLSI[a] %S / %R	ECV[b,c] %WT / %NWT
C. albicans (1405)					
Anidulafungin	0.03	0.06	≤0.008 – 0.5	99.6 / 0.0	99.1 / 0.9
Caspofungin	0.03	0.12	≤0.008 – 2	99.4 / 0.2	98.5 / 1.5
Micafungin	0.03	0.03	≤0.008 – 1	99.6 / 0.1	92.0 / 8.0
Fluconazole	0.12	0.25	≤0.06 – >128	99.5 / 0.4	98.2 / 1.8
Itraconazole	0.03	0.12	≤0.008 – >8	97.5 / 0.4	97.5 / 2.5
Posaconazole	0.03	0.06	≤0.008 – >8	- / -	92.2 / 7.8
Voriconazole	≤0.008	0.015	≤0.008 – >8	99.6 / 0.4	99.4 / 0.6
Amphotericin B	1	1	0.25 – 2	- / -	100.0 / 0.0
Flucytosine	≤0.5	1	≤0.5 – >32	97.8 / 2.1	89.2 / 10.8
C. glabrata (571)					
Anidulafungin	0.06	0.12	0.015 – 4	93.7 / 1.8	98.2 / 1.8
Caspofungin	0.03	0.12	0.015 – 16	96.0 / 1.6	96.0 / 4.0
Micafungin	0.015	0.03	≤0.008 – 2	97.9 / 1.2	95.8 / 4.2
Fluconazole	8	32	0.12 – >128	- / 8.8	91.2 / 8.8
Itraconazole	1	2	0.06 – >8	0.9 / 58.8	93.2 / 6.8
Posaconazole	1	2	0.06 – >8	- / -	96.3 / 3.7
Voriconazole	0.12	1	≤0.008 – 8	- / -	89.5 / 10.5
Amphotericin B	1	1	≤0.12 – 2	- / -	100.0 / 0.0
Flucytosine	≤0.5	≤0.5	≤0.5 – >32	99.1 / 0.4	98.4 / 1.6
C. parapsilosis (565)					
Anidulafungin	2	4	0.03 – 8	86.4 / 0.5	99.5 / 0.5
Caspofungin	0.25	0.5	0.03 – 2	100.0 / 0.0	99.6 / 0.4
Micafungin	1	2	0.06 – 4	99.5 / 0.0	100.0 / 0.0
Fluconazole	0.5	2	≤0.06 – >128	94.7 / 3.0	94.7 / 5.3
Itraconazole	0.12	0.25	≤0.008 – 1	73.3 / 1.1	98.9 / 1.1
Posaconazole	0.06	0.25	0.015 – 0.5	- / -	97.7 / 2.3
Voriconazole	0.015	0.06	≤0.008 – 4	97.2 / 0.2	97.2 / 2.8
Amphotericin B	1	1	0.25 – 2	- / -	100.0 / 0.0
Flucytosine	≤0.5	≤0.5	≤0.5 – >32	98.9 / 1.1	97.2 / 2.8
C. tropicalis (318)					
Anidulafungin	0.03	0.06	≤0.008 – 0.25	100.0 / 0.0	99.7 / 0.3
Caspofungin	0.03	0.06	≤0.008 – 0.25	100.0 / 0.0	99.1 / 0.9
Micafungin	0.03	0.06	≤0.008 – 0.25	100.0 / 0.0	99.4 / 0.6
Fluconazole	0.25	1	≤0.06 – 32	96.5 / 1.3	96.5 / 3.5
Itraconazole	0.06	0.25	≤0.008 – 1	89.3 / 1.6	98.4 / 1.6
Posaconazole	0.06	0.12	≤0.008 – 0.5	- / -	94.7 / 5.3
Voriconazole	0.015	0.06	≤0.008 – 1	96.2 / 0.3	91.8 / 8.2
Amphotericin B	1	1	0.25 – 2	- / -	100.0 / 0.0
Flucytosine	≤0.5	1	≤0.5 – >32	92.5 / 7.5	88.7 / 11.3
C. krusei (79)					
Anidulafungin	0.06	0.12	≤0.008 – 0.25	100.0 / 0.0	98.7 / 1.3
Caspofungin	0.12	0.25	≤0.008 – 0.5	94.9 / 0.0	94.9 / 5.1
Micafungin	0.12	0.12	≤0.008 – 0.5	98.7 / 0.0	98.7 / 1.3
Fluconazole	32	64	4 – 128	- / -	96.2 / 3.8
Itraconazole	0.5	1	0.06 – 2	5.1 / 29.1	97.5 / 2.5
Posaconazole	0.5	1	0.06 – 2	- / -	84.8 / 15.2
Voriconazole	0.25	0.5	0.06 – 2	93.7 / 1.3	93.7 / 6.3
Amphotericin B	1	2	0.5 – 2	- / -	100.0 / 0.0
Flucytosine	16	32	1 – >32	8.9 / 20.3	96.2 / 3.8
C. neoformans (84)					
Anidulafungin	>16	>16	>16	- / -	- / -
Caspofungin	>16	>16	8 – >16	- / -	- / -
Micafungin	>16	>16	>16	- / -	- / -
Fluconazole	4	8	0.5 – 16	- / -	97.6 / 2.4
Itraconazole	0.12	0.5	0.03 – 1	- / -	- / -
Posaconazole	0.12	0.5	0.015 – 1	- / -	83.3 / 16.7
Voriconazole	0.03	0.06	≤0.008 – 0.12	- / -	100.0 / 0.0
Amphotericin B	1	1	0.25 – 1	- / -	100.0 / 0.0
Flucytosine	4	8	≤0.5 – >32	- / -	91.6 / 8.4
A. fumigatus (97)					
Anidulafungin	0.015	0.03	≤0.008 – 0.06	- / -	- / -
Caspofungin	0.03	0.03	≤0.008 – 0.06	- / -	- / -
Micafungin	≤0.008	0.015	≤0.008 – 0.03	- / -	- / -
Fluconazole	>128	>128	>128	- / -	- / -
Itraconazole	1	1	0.5 – 2	- / -	97.9 / 2.1
Posaconazole	0.25	0.5	0.25 – 4	- / -	90.6 / 9.4
Voriconazole	0.5	0.5	0.25 – 8	- / -	99.0 / 1.0
Amphotericin B	2	>2	1 – >2	- / -	87.6 / 12.4
Flucytosine	>32	>32	16 – >32	- / -	- / -

a. Clinical Laboratory and Standards Institute (CLSI), susceptible (S), resistant (R).
b. Epidemiologic cutoff value (ECV), wild-type (WT), non wild-type (NWT).
c. According to Pfaller et al. 2010 and 2011 (for *Candida* spp. and *C. neoformans*). According to Espinel-Ingroff et al., 2010 and 2011 (for *Aspergillus* spp.).

Table 2. Antifungal activity of echinocandins against five most common *Candida* species.

Organism (no.tested)/ antimicrobial agent	Number (cumulative %) of isolates inhibited at MIC (µg/ml) ≤0.03	0.06	0.12	0.25	0.5	1	2	4	8
C. albicans (1405)									
Anidulafungin	947 (67.3)	347 (92.0)	99 (99.1)	7 (99.6)	5 (100.0)				
Caspofungin	880 (62.6)	364 (88.5)	140 (98.5)	13 (99.4)	5 (99.7)	2 (99.9)	1 (100.0)		
Micafungin	1293 (92.0)	88 (98.3)	15 (99.4)	3 (99.6)	4 (99.9)	2 (100.0)			
C. glabrata (571)									
Anidulafungin	91 (15.9)	297 (67.9)	147 (93.7)	26 (98.2)	2 (98.6)	6 (99.6)	1 (99.8)	1 (100.0)	
Caspofungin	341 (59.7)	133 (83.0)	74 (96.0)	14 (98.4)	3 (99.0)	4 (99.7)	0 (99.7)	1 (99.8)	1 (100.0)
Micafungin	547 (97.0)	12 (97.9)	5 (98.8)	1 (99.0)	4 (99.7)	1 (99.8)	1 (100.0)		
C. parapsilosis (565)									
Anidulafungin	1 (0.2)	0 (0.2)	3 (0.7)	24 (5.0)	57 (15.0)	181 (47.1)	222 (86.4)	74 (99.5)	3 (100.0)
Caspofungin	2 (0.4)	8 (1.8)	59 (12.2)	296 (64.6)	148 (90.8)	50 (99.6)	2 (100.0)		
Micafungin	0 (0.0)	1 (0.2)	5 (1.1)	14 (3.5)	76 (17.0)	299 (69.9)	167 (99.5)	3 (100.0)	
C. tropicalis (318)									
Anidulafungin	279 (87.7)	34 (98.4)	4 (99.7)	1 (100.0)					
Caspofungin	246 (77.4)	54 (94.3)	15 (99.1)	3 (100.0)					
Micafungin	255 (80.2)	55 (97.5)	6 (99.4)	2 (100.0)					
C. krusei (79)									
Anidulafungin	32 (40.5)	36(86.1)	10 (98.7)	1 (100.0)					
Caspofungin	3 (3.8)	24 (34.2)	17 (55.7)	31 (94.9)	4 (100.0)				
Micafungin	2 (2.5)	22 (30.4)	54 (98.7)	1 (100.0)					

Table 3. Summary of FKS alterations detected in echinocandin-resistant *Candida* spp. strains.

Isolate	Site Code	Year	Organism	State and/or Country	MIC (µg/ml): Anidulafungin	Caspofungin	Micafungin	1,3-β-D-glucan synthase alterations *FKS1* HS1	*FKS1* HS2	*FKS2* HS1	*FKS2* HS2
65388f	002	2010	*C. glabrata*	Indiana, USA	1	4	0.06	WT[a]	WT	F641V	WT
29874f	131	2010	*C. glabrata*	Belgium	1	1	0.06	WT	WT	WT	WT
30011f	003	2010	*C. glabrata*	Michigan, USA	0.25	0.5	0.03	WT	WT	D648E	WT
35441f	024	2010	*C. glabrata*	Texas, USA	0.5	0.25	0.03	WT	WT	F641Y	WT
48262A	088	2010	*C. glabrata*	Germany	1	0.5	0.5	WT	WT	L644W	WT
53301f	141	2010	*C. glabrata*	New York, USA	0.25	0.25	0.5	WT	WT	WT	WT
19022f	002	2010	*C. parapsilosis*	New York, USA	8	2	1	WT	WT	-	-
19030f	002	2010	*C. parapsilosis*	New York, USA	8	1	1	WT	WT	-	-
19471f	039	2010	*C. parapsilosis*	Argentina	8	1	1	WT	WT	-	-
43574f	089	2011	*C. albicans*	Sweden	0.5	1	1	S654P	neg	-	-
50733f	339	2011	*C. albicans*	Scotland	0.5	2	1	S629P	neg	-	-
52537f	230	2011	*C. albicans*	China	0.25	1	0.5	S645P	neg	-	-
49117f	203	2011	*C. glabrata*	Australia	0.5	0.25	0.12	F625S	neg	neg	neg
49885f	147	2011	*C. glabrata*	Canada	1	1	0.25	neg	neg	S659Y	neg
37706f	002	2011	*C. glabrata*	Indiana, USA	1	0.5	0.5	neg	neg	S663Y	neg
49079f	266	2011	*C. glabrata*	Australia	1	1	0.5	neg	neg	S663P	neg
45049f	062	2011	*C. glabrata*	Greece	2	1	1	neg	neg	S663P	neg
42568F	448	2011	*C. glabrata*	Louisiana, USA	4	16	2	S629P	neg	neg	neg

a. WT= wildtype; b. "-"= not tested.

Conclusions

- Resistance rates for echinocandins and triazoles remains very low among *Candida* species; however, emergence of echinocandin-resistance among *C. albicans* and cross-resistance for echinocandins and azoles among *C. glabrata* strains warrants further surveillance.
- Mutations leading to changes in the *FKS* were detected among all echinocandin-resistant *C. albicans* and most of the *C. glabrata* strains analyzed; however, *C. parapsilosis* harbored changes in those genes regardless of the elevated MIC values noted.

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