Low Prevalence of *fks1* Hotspot 1 Mutations in a Worldwide Collection of *Candida* spp.

M-1724

LN WOOSLEY, M CASTANHEIRA, MA PFALLER, D DIEKEMA, S MESSER, RN JONES
JMI Laboratories, North Liberty, IA, USA; University of Iowa, Iowa City, IA, USA

ICAAC 2009
JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
319.665.3370, 319.665.3371
mariana-castanheira@jmilabs.com

ABSTRACT

Background: Echinocandins (ECH) are valuable treatment options for invasive fungal infections due to low toxicity, infrequent side effects and lack of cross-resistance with azoles. Reduced ECH susceptibility (S) in *Candida* spp. (CSP) have been primarily associated with mutations in the hot spot (HS) 1 *fks1* subunit of 1,3 β-D-glucan synthase. We evaluated the prevalence of *fks1* HS1 mutations among CSP (6 species) displaying a range of ECH MIC values.

Methods: 134 CSP isolates (6 species) from a worldwide surveillance collection were S tested by broth microdilution according to CLSI guidelines. DNA preparations were used for amplification of *fks1* HS1. Amplicons were sequenced and analyzed. Species identification was confirmed by ITS sequencing, when necessary.

Results: Among 134 CSP tested displaying various caspofungin (CASP; [ECH class surrogate]) MIC values (ranging from ≤0.008->16 µg/ml), 4 FKS1 HS1 aminoacid (aa) substitutions related to ECH resistance were detected (3.0% overall). These were observed in 1 *C. albicans* ([CA] F641Y) out of 32 isolates tested (3.1%), 1 *C. glabrata* ([CGLA] S645P) among 34 isolates (2.9%), and 2 *C. tropicalis* ([CTRO] F641S) of 12 strains evaluated (16.7%). The 4 isolates displaying FKS1 HS1 alterations showed elevated CASP MIC results (1, 8, 16, 1 µg/ml for CA, CGLA, 2 CTRO, respectively). Genetic polymorphisms were observed in the diploid species (CA and *C. krusei* [CK]), without aa alterations. CK, *C. parapsilosis*, and *C. guilliermondii* isolates tested showed no FKS1 HS1 alterations.

Species (No. tested)	CASP MIC range (µg/ml)	Number of strains with FKS1 HS1 aa alterations	Number of strains with *fks1* HS1 polymorphism
C. albicans (32)	≤0.008-1	1 (F641Y)	13 A/T1929 2 C/T1923
C. krusei (11)	0.03-1	0	5 A/G2034
C. glabrata (34)	≤0.016-8	1 (S645P)	0
C. tropicalis (12)	≤0.016-16	2 (F641S)	0
C. parapsilosis (25)	0.06-4	0	0
C. guilliermondii (20)	0.25->16	0	0

Conclusions: Results from this large, geographically diverse CSP collection demonstrated that *fks1* HS1 mutations remain uncommon among isolates with various ECH MIC levels.

INTRODUCTION

Candida species are the most common cause of invasive fungal infections among hospitalized patients, accounting for 8 to 10% of all nosocomial bloodstream infections. In addition, invasive candidiasis is associated with very high crude and attributable mortality rates. Treatment can be challenging with several options available differing in antifungal cost and toxicity.

The introduction of echinocandin compounds was an important advance in the treatment of invasive fungal infections, providing a well tolerated and effective alternative to azoles and polyenes. Echinocandins are often used for primary therapy for invasive candidiasis, based upon a favorable drug interaction profile, low toxicity, and good activity against species that may demonstrate resistance or reduced susceptibility to azoles and polyenes (e.g. *C. glabrata*, *C. krusei*).

Echinocandins are lipopeptides that inhibit cell wall synthesis by targeting the 1,3-β-D-glucan synthase complex. Resistance to these drugs has been associated with mutations within two highly conserved regions of *fks1* and *fks2*. Amino acid substitutions in the proteins encoded by these genes can occur within two hot spots (HS) on each gene. Reduced echinocandin susceptibility has primarily been associated with mutations in the HS1 region of *fks*1. In this study, we evaluated 134 *Candida* spp. isolates for mutations within HS1 of *fks1*. A subset of *C. albicans* (8 strains) and *C. glabrata* (7 strains) were evaluated for mutations on other HSs of *fks1* and *fks2*.

MATERIALS AND METHODS

Isolates. A total of 134 *Candida* spp. clinical isolates showing caspofungin MIC values ranging from ≤0.008 to 16 µg/ml were evaluated. The isolates included two groups, one subset with lower echinocandin MIC values (Wildtype [WT]) and one group with reduced susceptibility against these compounds (non-WT). These *Candida* spp. strains belonged to 6 species, including: *C. glabrata* (34), *C. albicans* (32), *C. parapsilosis* (25), *C. guilliermondii* (20), *C. tropicalis* (12) and *C. krusei* (11). These strains were acquired from worldwide surveillance collections: the SENTRY Antimicrobial Surveillance Program and ARTEMIS.

Isolates were identified at the participating medical centers by the established methods in use at each institution. Confirmation of species identification was performed at the central reference laboratory using Vitek (bioMérieux, Missouri, USA), conventional reference methods and/or 28S and ITS sequencing as described elsewhere.

Antimicrobial susceptibility testing. Broth microdilution MIC testing was performed according to Clinical and Laboratory Standards Institute (CLSI) methods (M27-A2; 2002). Panels were produced using RPMI 1640 broth supplemented with MOPS (morpholinepropane-sulfonic acid) buffer. All results were recorded at 24 and 48 hours and the interpretive criteria used were those published in CLSI document M27-S2 (2006). Quality control (QC) was performed as recommended in M27-A2 (2002) using the following QC strains: *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258.

fks sequencing. DNA extraction was performed using QIAamp DNA mini kit (Qiagen, Hilden, Germany). Singleplex PCR reactions were set up with generic *fks* primers (Table 1) that were able to amplify most *Candida* species. *C. glabrata* and *C. krusei* required species-specific *fks* primers. PCR 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). Sequences were compared to other available via internet sources (http://www.ncbinlm.nih.gov/blast/).

RESULTS

- Isolates were collected from medical centers located in North America (41.0% of the strains), Europe (23.9%), Asia-Pacific Region (22.4%) and Latin America (12.7%).
- MIC distributions for echinocandins compounds tested against 134 strains showed that 14 *C. glabrata*, 4 *C. krusei*, 3 *C. guilliermondii*, 2 *C. tropicalis* and 1 *C. albicans* had a caspofungin MIC higher than the wildtype (WT) population (Table 2).
- Four (2.9%) *Candida* strains displayed *fks1* HS1 mutation: 1 *C. albicans* (F641Y), 1 *C. glabrata* (S645P), and 2 *C. tropicalis* (F641S; Figure 1).
- *C. glabrata* and *C. tropicalis* strains exhibiting *fks1* HS1 mutations demonstrated elevated MIC values for caspofungin, anidulafungin and micafungin (0.5->8 µg/ml, Table 3).
- *C. albicans* showed modestly elevated MIC values for caspofungin (1 µg/ml), but routinely had lower MIC values for anidulafungin and micafungin (0.12 and 0.25 µg/ml, respectively).
- Genetic polymorphisms in *fks1* HS1 were detected in the diploid species *C. albicans* (A/T1929) and *C. krusei* (A/G2034) among 47 and 45% of the isolates, respectively.
- No *fks1* HS2 mutations were found in the 8 *C. albicans* strains selected.
- Among 7 *C. glabrata* strains tested for mutations of HS2 *fks1,* and HS1 and HS2 of *fks2*, 3 isolates displayed mutations that encoded aminoacid alterations within *FKS2* HS1 (S645P and L644W; Figure 1).
- Two *C. glabrata* with *fks* mutations in *fks2* HS1 produced non-susceptible MIC values for all echinocandin compounds. The remaining strain had lower MIC values for these agents (Table 2).

Table 1. Oligonucleotides used in this study.

Region	Oligo name	Sequence (5' to 3')
fks1 HS1	fks-CSP-F	ATT GGG CTG GTG CTC AAC AT
	fks-CSP-R	CCT TCA ATT TCA GAT GGA ACT TGA TG
	CKRUfksF	ACT GCA TCG TTT GCT
	CKRUfksR	GAA CAT GAT CAA TTG CCA AC
	CGLAfks-F	CCA TTG GGT GGT CTG TTC ACG
	CGLAfks-R	GAT TGG GCA AAG AAA GAA ATA CGA C
fks1 HS2	CSP-fks1HS2-F	AAG ATT GGT GCT GGT ATG GG
	CSP-fks1HS2-R	TAA TGG TGC TTG CCA ATG AG
	CGLA-fks1HS2-R	ATG GAG AGA ACA GCA GGG CG
fks2 HS1	CGLA-fks2HS1-F	GCT TCT CAG ACT TTC ACC G
	CGLA-fks2HS1-R	CAG AAT AGT GTG GAG TCA AGA CG
fks2 HS2	CGLA-fks2HS2-F	TCT TGA CTT TCT ACT ATG CG
	CGLA-fks2HS2-R	CTT GCC AAT GTG CCA CTG

Figure 1. Alignments of FKS hot spot regions for amino acid alterations observed in this study. Amino acid positions are defined as equivalent to those for *C. albicans*.

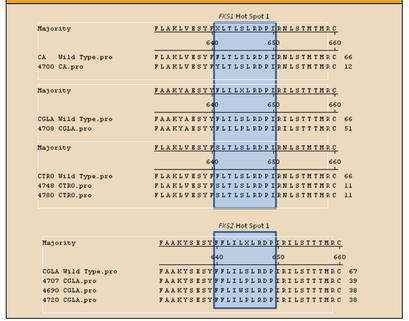

Table 2. MIC distributions of echinocandin compounds against 134 *Candida* spp. strains tested for the presence of *fks* mutations.

Organism (no. of strains)	MIC in µg/ml (no. of strains)[a,b,c]											
	≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	>8
C. glabrata (34)												
Anidulafungin	-	-	7	15	4 (1)	2	-	3	-	3 (3)	-	-
Caspofungin	-	-	1	19	2	1 (1)	5	1	2	2	1 (1)	2 (2)
Micafungin	2	21	2	1 (1)	2	2	1	-	1 (1)	2 (2)	-	-
C. albicans (32)												
Anidulafungin	3	10	13	4	2 (1)	-	-	-	-	-	-	-
Caspofungin	3	2	9	17	-	-	-	1 (1)	-	-	-	-
Micafungin	3	21	7	-	-	1 (1)	-	-	-	-	-	-
C. parapsilosis (25)												
Anidulafungin	-	-	-	-	-	2	2	5	13	3	-	-
Caspofungin	-	-	-	-	-	1	14	10	-	-	-	-
Micafungin	-	-	-	-	-	2	3	3	15	2	-	-
C. guilliermondii (20)												
Anidulafungin	-	-	-	-	-	-	-	1	10	9	-	-
Caspofungin	-	-	-	-	-	1	1	13	2	2	1	-
Micafungin	-	-	-	-	-	-	4	7	7	2	-	-
C. tropicalis (12)												
Anidulafungin	1	6	3	-	-	-	-	2 (2)	-	-	-	-
Caspofungin	-	2	6	2	-	-	-	-	-	2 (2)	-	-
Micafungin	1	2	6	1	-	-	2 (2)	-	-	-	-	-
C. krusei (11)												
Anidulafungin	-	1	3	7	-	-	-	-	-	-	-	-
Caspofungin	-	-	1	4	-	1	1	3	1	-	-	-
Micafungin	-	-	-	8	3	-	-	-	-	-	-	-

a. Shaded areas correspond to the wildtype population.
b. Epidemiological cutoff values (ECV) are boxed (Pfaller et al., 2009).
c. Numbers in parenthesis shows positions of the strains demonstrating *fks* mutations.

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

Isolate	Species	Alterations		MIC (µg/ml)			Location
		fks1 HS1	*fks2* HS1	Caspofungin	Anidulafungin	Micafungin	
4700	*C. albicans*	F641Y	NT[a]	1	0.12	0.25	New York, NY
4748	*C. tropicalis*	F641S	NT	4	1	0.5	Akron, OH
4780	*C. tropicalis*	F641S	NT	4	1	0.5	Akron, OH
4708	*C. glabrata*	S645P	NT	≥8	4	4	Akron, OH
4707	*C. glabrata*	NM[b]	S645P	8	4	2	Cleveland, OH
4690	*C. glabrata*	NM	L644W	0.25	0.12	0.06	Japan
4720	*C. glabrata*	NM	S645P	≥8	4	4	Detroit, MI

a. NT = Not Tested.
b. NM = No Mutation.

- All strains showing *fks* mutations demostrated elevated caspofungin MIC results (Table 2), however; two strains displayed anidulafungin and/or micafungin MIC values within the WT population for these echinocandins.
- *Candida* spp. strains harboring *fks* HS mutations demonstrated MIC values for echinocandins at or greater than recently established epidemiologic cutoff values (ECV; Table 1).

CONCLUSIONS

- Two of the amino acid alterations detected among *C. glabrata* (*fks1* HS1 F641S and *fks2* HS1 L644W) strains were previously reported only from *C. albicans* and *C. tropicalis*.
- Mutations on *fks2* HS1 were detected in *C. glabrata*, however one of the strains showed very low MIC values for echinocandins.
- *Candida* spp. strains showing caspofungin MIC values corresponding to WT distribution did not have *fks* mutations. In contrast, strains showing *fks* HS mutations displayed anidulafungin and/or micafungin MIC values within the WT population, suggesting that caspofungin could be the most sensitive agent for detection of these resistance mutations.
- *Candida* spp. isolates showing distinct echinocandin MIC values were selected from worldwide collections and tested for the presence of *fks1* HS1 mutations. Results indicate that these mutations of this region appear to be uncommon.

REFERENCES

1. Clinical and Laboratory Standards Institute (2008). *M27-S3. Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeasts. 3rd Informational Supplement*. Wayne, PA: CLSI.
2. Desnos-Ollivier M, Bretagne S, Raoux D, Hoinard D, Dromer F, Dannaoui E (2008). Mutations in the fks1 gene in *Candida albicans, C. tropicalis*, and *C. krusei* correlate with elevated caspofungin MICs uncovered in AM3 medium using the method of the European Committee on Antibiotic Susceptibility Testing. *Antimicrob Agents Chemother* 52: 3092-3098.
3. Katiyar S, Pfaller M, Edlind T (2006). *Candida albicans* and *Candida glabrata* clinical isolates exhibiting reduced echinocandin susceptibility. *Antimicrob Agents Chemother* 50: 2892-2894.
4. Leaw SN, Chang HC, Sun HF, Barton R, Bouchara JP, Chang TC (2006). Identification of medically important yeast species by sequence analysis of the internal transcribed spacer regions. *J Clin Microbiol* 44: 693-699.
5. Mills EJ, Perri D, Cooper C, Nachega JB, Wu P, Tleyjeh I, Phillips P (2009). Antifungal treatment for invasive *Candida* infections: a mixed treatment comparison meta-analysis. *Ann Clin Microbiol Antimicrob* 8: 23.
6. Pfaller MA, Boyken L, Hollis RJ, Kroeger J, Messer SA, Tendolkar S, Jones RN, Diekema D (2009). Wild-type MIC distributions and epidemiological cutoff values (ECVs) for the echinocandins and *Candida* spp. *J Clin Microbiol Submitted*: