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Detection of *fks* gene Hot Spot Mutations in *Candida glabrata* by CLSI Broth Microdilution Testing of Anidulafungin, Caspofungin and Micafungin Using Epidemiological Cutoff Values

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ABSTRACT

Background: *C. glabrata* (CG) is a leading human fungal pathogen that can rapidly evolve resistance to echinocandins via the acquisition of point mutations in hot spot (HS) regions of the *fks* genes. A 10-year study by a single USA medical center found that the presence of an elevated MIC for caspofungin (CSF; MIC, >0.12 µg/mL) or micafungin (MCF; MIC, >0.06 µg/mL) served as a sensitive screen for the presence of clinically significant mutations in *fks*. In the present study, we extend these observations using a collection of CG isolates from 2 global surveys.

Methods: Previously defined epidemiological cutoff values (ECVs) were used to classify isolates as either wildtype (WT; MIC ≤ ECV) or non-WT (MIC > ECV) to anidulafungin (ANF; ECV at 0.25 µg/mL), CSF (ECV at 0.12 µg/mL) and MCF (ECV at 0.03 µg/mL). A total of 119 clinical isolates of CG representing both WT and non-WT echinocandin MICs were tested against ANF, CSF, and MCF using CLSI BMD methods. The HS1/HS2 regions of *fks1* and *fks2* were sequenced for all isolates. The *fks3* was also sequenced for all strains and the entire *fks1* and *fks2* was sequenced for 22 selected isolates.

Results: Among the 119 isolates, 31 (26%) were classified as non-WT to MCF, 23 (19%) to ANF, and 39 (33%) to CSF. Mutations in *fks* HSs were detected in a total of 28 strains: 24 (86%) were non-WT for MCF, 22 (79%) for ANF, and 25 (89%) for CSF. Isolates carrying *fks* HS mutations were detected in 7 different countries and eight USA states. Overall, 22 (79%) of the *fks* mutants had non-WT MIC values for at least one echinocandin and three had WT MICs for all three echinocandins (89% concordance across all three agents). The most common mutations for those strains with non-WT MICs were at positions F659 (7 isolates, 3 substitutions) and S663 (7 isolates, 3 substitutions). The three *fks* mutants for which MIC results were WT for all echinocandins had substitutions of F625Y, L630I, and D632Y. 9/22 CG strains had non-HS mutations on *fks1* and/or *fks2*. No isolates harbored *fks3* mutations.

Conclusions: These results confirm those of previous single-center studies and demonstrate comparable ability of BMD MIC testing with any of the three echinocandins to detect clinically important mutations in *fks* genes.

INTRODUCTION

Candida glabrata is a leading human fungal pathogen causing life-threatening infections that has intrinsically elevated MIC values to the azole agents; it additionally has the ability to develop resistance to azoles and echinocandins. Echinocandin resistance in *C. glabrata* is commonly caused via the acquisition of point mutations in hot spot (HS) regions of the *fks* gene encoding the 1,3 β-D-glucan synthase (GS), target of the echinocandin class.

Due to the spectrum and fungicidal potency against *Candida* spp., the echinocandin antifungal agents (anidulafungin, caspofungin, and micafungin) are recommended as first-line therapy for most patients with invasive candidiasis, followed by de-escalation to fluconazole based on the susceptibility of the infecting organism to fluconazole. Resistance to the systemically active echinocandin and azole antifungal agents is distinctly uncommon among bloodstream infection isolates of *C. albicans, C. tropicalis, C. parapsilosis,* and *C. krusei* (intrinsically resistant to fluconazole, but susceptible to the echinocandins, voriconazole and posaconazole), but the emergence of resistance to both classes of agents is now evident in *C. glabrata* suggesting that this species requires greater monitoring for resistance. Furthermore, a 10-year study by a single USA medical center found that the presence of an elevated MIC for caspofungin (MIC, >0.12 µg/mL) or micafungin (MIC, >0.06 µg/mL) served as a sensitive screen for the presence of clinically significant mutations in *fks*.

In the present study, we evaluate 119 wildtype and non-wildtype *C. glabrata* strains collected during two global surveillance programs from 2001 to 2011. Isolates were re-tested by the Clinical and Laboratory Standards Institute (CLSI) reference broth microdilution and were evaluated for the presence of *fks* mutations.

MATERIALS AND METHODS

Organisms. A total of 119 *C. glabrata* isolates collected during the ARTEMIS and SENTRY surveillance programs from 2001 to 2011 displayed MIC values potentially outside of the wildtype MIC distribution for the echinocandins upon initial testing and were selected for further analysis. These isolates initially displayed micafungin MICs at ≥0.06 µg/mL and/or anidulafungin MICs at ≥0.25 µg/mL; and/or caspofungin MICs at ≥0.25 µg/mL and were distributed among both surveillance studies and throughout the years monitored.

Susceptibility testing. Broth microdilution MIC testing was performed according to CLSI methods (M27-A3; 2008). Panels were produced by JMI Laboratories (North Liberty, Iowa, USA) using RPMI 1640 broth supplemented with MOPS (morpholinepropane-sulfonic acid) buffer. Results were read visually after 24-h incubation as the lowest concentration producing prominent inhibition (≥50%) of growth relative to the drug-free control for the echinocandins (anidulafungin, caspofungin and micafungin) and azoles (fluconazole, posaconazole and voriconazole) and the interpretive criteria used were those published in CLSI document M27-S4 (2012) and/or by Pfaller et al (2011). Quality control (QC) was performed as recommended in M27-A3 (2008) using the following QC strains: *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258.

fks sequencing. DNA extraction of the *C. glabrata* isolates was performed using the Qiagen QIAamp DNA mini kit (Qiagen, Hilden, Germany) in the QiaCube automated platform (Qiagen). PCR amplification was carried on using previously described oligunucleotides for the HS and *fks3*. Custom designed primers used to obtain the entire sequence of *fks1* and *fks2*. Amplicons were sequenced on both strands and the nucleotide sequences and deduced amino acid sequences were analyzed using the Lasergene software package (DNASTAR, Madison, Wisconsin). Sequences were compared to those of *C. glabrata* ATCC MYA-2950.

RESULTS

- Mutations on the HS of *fks* genes were detected among 28 strains with FKS1 HS1 and FKS2 HS1 the most common (eight and 19 strains, respectively). One isolate had an FKS2 HS2 (P1371S) and no strains carried mutations on *fks1* HS2 or *fks3*.
- FKS1 HS1 S629P alteration (four strains) and FKS2 HS1 S663P (four strains) were the most common substitutions; and 14 (51.8% of the strains carrying mutations) strains had substitutions in positions S663 and F659 (seven each) of FKS2 HS1 (**Table 1**).
- One strain had two HS mutations (S629P + R631S) and displayed a micafungin MIC of 16 µg/mL, anidulafungin of 2 µg/mL and caspofungin of 4 µg/mL.
- Isolates carrying mutations on *fks* HSs were detected in seven countries and were more common in USA (16 strains; eight states) and Germany (five). Eleven (39.3%) of these strains were also resistant to fluconazole.
- FKS non-HS alterations were detected among 13 of 22 strains, four also harboring HS substitutions.
- All isolates displaying non-HS mutations had micafungin MIC values of <0.12 µg/mL and were categorized as susceptible (**Table 2**, **Figure 1**). Eight strains carrying non-HS substitutions were categorized as intermediate for caspofungin (MIC, 0.25 µg/mL; **Table 2**).
- Nine isolates without mutations on *fks* genes had micafungin MIC values <0.12 µg/mL, and anidulafungin of <0.25 µg/mL, but caspofungin MICs ranging from 0.06 to 0.25 µg/mL (**Figure 1**).
- All isolates categorized as micafungin-intermediate (five strains) or -resistant (15; MIC, >0.12 µg/mL) displayed HS mutations and the highest MIC values were achieved by the presence of two HS mutations (**Figure 1**). For anidulafungin and caspofungin, 22 of 23 (95.7%) and 19 of 19 (100.0%) among the resistant (MIC, >0.25 µg/mL) strains harbored HS substitutions, but intermediate strains showed mutations in 3 of 4 (75.0%) or 13 of 19 (68.4%) isolates, respectively.

Table 1. *FKS* hotspot alterations in isolates of *C. glabrata* from the ARTEMIS and SENTRY Surveillance Programs, 2001-2011.

FKS alterations[a]	Year	State and/or Country	MIC (µg/mL)			
			Micafungin	Anidulafungin	Caspofungin	Fluconazole
FKS1 HS1						
F625Y	2010	Texas	0.03	0.25	0.12	16
F625S	2011	Australia	0.12	1	0.5	128
S629P	2008	Ohio	1	2	16	128
S629P	2008	Washington	0.5	2	16	32
S629P	2011	Louisiana	4	2	16	128
S629P, R631S	2008	Indiana	16	4	2	8
L630I	2009	Massachusetts	0.015	0.03	0.03	4
D632Y	2008	Washington	0.015	0.25	0.12	128
FKS1 HS2						
F659S	2006	Germany	0.06	0.5	0.5	16
F659S	2007	Indiana	0.06	0.25	0.25	16
F659S	2009	Germany	0.06	1	0.5	128
F659Y	2003	Japan	0.25	1	8	2
F659V	2006	Virginia	0.12	1	2	128
F659Y	2005	Ohio	0.12	1	1	4
F659Y	2011	Canada	0.25	1	2	16
L662W	2004	Japan	0.5	2	1	64
L662W	2010	Germany	0.25	1	0.5	2
S663P	2008	Germany	0.25	1	1	64
S663P	2008	Indiana	4	4	16	4
S663P	2011	Australia	2	2	2	128
S663P	2011	Greece	2	4	4	16
S663F	2008	Washington	0.12	0.5	0.25	4
S663F	2010	Ohio	0.12	0.5	0.25	4
S663Y	2011	Indiana	0.5	1	0.5	8
L664R	2004	Spain	0.25	1	0.25	128
L664R	2004	Spain	0.25	1	0.5	64
D666Y	2005	Germany	0.06	0.12	0.25	8
FKS2 HS2						
P1371S	2011	New York	0.03	0.12	0.25	4

a. Amino acid substitutions of FKS1 HS1 or FKS3 HS1 were not detected.

Table 2. *C. glabrata* strains from the ARTEMIS and SENTRY Surveillance Programs, 2001-2011 displaying non-HS FKS alterations.

Isolate	Year	State and/or Country	MIC (µg/mL)				FKS1 HS1	FKS2 HS1	FKS2 HS2	Non-HS	
			Micafungin	Anidulafungin	Caspofungin	Fluconazole				FKS1	FKS2
46228A	2011	South Africa	0.015	0.06	0.25	128	neg	neg	neg	Y1279F	T926P
49889F	2011	Canada	0.015	0.03	0.25	32	neg	neg	neg	neg	G680S
10729J	2004	UK	0.015	0.06	0.25	1	neg	neg	neg	L195F	neg
10685J	2004	Spain	0.015	0.06	0.25	32	neg	neg	neg	neg	F384Y
10702J	2005	Spain	0.015	0.12	0.25	16	neg	neg	neg	A995T	neg
10728J	2002	Spain	0.015	0.06	0.25	1	neg	neg	neg	D140Y, A1749V	neg
44075F	2011	WA, USA	0.03	0.06	0.12	32	neg	neg	neg	neg	T926P
17823F	2011	MI, USA	0.06	0.12	0.06	128	neg	neg	neg	neg	A450T
10708J	2005	Poland	0.06	0.25	0.25	16	neg	neg	neg	A1749V	neg
760F	2008	WA, USA	0.12	0.5	0.25	4	neg	S663F	neg	neg	T926P
37706F	2011	IN, USA	0.5	1	0.5	8	neg	S663Y	neg	neg	G680S
10700J	2004	Japan	0.5	2	1	64	neg	L662W	neg	neg	E78D, T926P
49079F	2011	Australia	2	2	2	128.1	neg	S663P	neg	neg	T926P

Figure 1. MIC distribution for echinocandins tested against 119 *C. glabrata* strains and FKS alterations in each MIC values as tested using the CLSI reference method.

Method	No. of isolates at MIC (µg/mL) (no. of isolates with FKS substitutions/no. of isolates with HS substitutions)[a,b]: ≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
Micafungin	6 (0/0)	63 (8/2)	19 (3/2)	9 (6/4)	7 (5/5)	6 (6/6)	3 (3/3)	1 (1/1)	2 (2/2)	2 (2/2)		1 (1/1)
FKS alterations (no. of isolates if >1)		**L630I** **D632Y** **D140Y+ A1749V** **L195F** **A995T** **Y1279P** +T926P G680S F384Y	**F625Y** P1371S T926P	D666Y F659S(3) **A1479V** A450T	**F625S** S663F S663F +T926P F649Y F649V	S663P L664R(2) L662W F659Y F659V	**S629P** S663Y +G680S L662W +E78D +T926P	**S629P**	S663P S663P +T926P	**S629P** S663P		**S629P +R631S**
Anidulafungin	3 (0/0)		11 (2/1)	40 (5/0)	32 (4/2)	10 (4/3)	3 (3/3)	12 (11/11)	5 (5/5)	3 (3/3)		
FKS alterations (no. of isolates if >1)			**L630I** G680S	**D140Y+ A1749V** **L195F** **Y1279P** +T926P F384Y T926P	D666Y P1371S A450T **A995T**	**D632Y** **F625Y** **A1479V** F659S	S663F S663F +T926P F659S	**F625S** F659S F659Y(2) F659V(2) L664R(2) L662W S663P +G680S	**S629P(2)** **S629P +R631S** S663P +T926P L662W +E78D +T926P	**S629P** S663(2)		
Caspofungin	1 (0/0)	20 (1/1)	34 (1/0)	25 (3/2)	19 (13/6)	6 (6/6)	3 (3/3)	4 (4/4)	1 (1/1)	1 (1/1)	4 (4/4)	
FKS alterations (no. of isolates if >1)		**L630I**	A450T	**D632Y** **F625Y** T926P	**D140Y+ A1749V** **L195F** **A995T** **A1749V** **Y1279P** +T926P F659S S663F L664R D666Y P1371S S663F +T629P G680S F384Y	F625S F659S(2) L664R L662W S663Y +G680S	F659Y S663P L662W +E78D +T926P	F649V F659Y **S629P +R631S** S663P +T926P	S663P	F659V	**S629P(3)** S663P

a. Fks1 alterations are bolded and hotspot (HS) alterations are underlined. Combination of amino acid substitutions in a single strain are boxed.
b. MIC values corresponding to the CLSI breakpoint criteria is highlighted in green for susceptible, yellow for intermediate and light red for resistant.

CONCLUSIONS

- Overall, *fks* mutations were detected in 41 (34.5%) *C. glabrata* strains from this 10-year worldwide collection; and only 28 (23.5%) had HS mutations.
- Among the echinocandin-susceptible population, 80 strains had no *fks* alterations and only eight (10.0%) strains displayed HS mutations. However, these specific mutations were not detected in intermediate/resistant strains.
- Using the current breakpoint criteria, micafungin would be a valuable surrogate predictor of relevant HS mutations to distinguish populations carrying HS and non-HS mutations.
- The role of mutations detected in echinocandin susceptible strains on clinical outcome is not known.

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