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SUSCEPTIBILITY

Echinocandins Susceptibility Patterns of 2,787 Yeast Isolates: Importance of the Thresholds for the Detection of FKS Mutations

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ABSTRACT Since echinocandins are recommended as first line therapy for invasive candidiasis, detection of resistance, mainly due to alteration in FKS protein, is of main interest. EUCAST AFST recommends testing both MIC of anidulafungin and micafungin, and breakpoints (BPs) have been proposed to detect echinocandin-resistant isolates. We analyzed MIC distribution for all three available echinocandins of 2,787 clinical yeast isolates corresponding to 5 common and 16 rare yeast species, using the standardized EUCAST method for anidulafungin and modified for caspofungin and micafungin (AM3-MIC). In our database, 64 isolates of common pathogenic species were resistant to anidulafungin, according to the EUCAST BP, and/or to caspofungin, using our previously published threshold (AM3-MIC $\geq$ 0.5 mg/L). Among these 64 isolates, 50 exhibited 21 different FKS mutations. We analyzed the capacity of caspofungin AM3-MIC and anidulafungin MIC determination in detecting isolates with FKS mutation. They were always identified using caspofungin AM3-MIC and the local threshold while some isolates were misclassified using anidulafungin MIC and EUCAST threshold. However, both methods misclassified four wild-type *C. glabrata* as resistant. Based on a large data set from a single center, the use of AM3-MIC testing for caspofungin looks promising in identifying non-wild-type *C. albicans*, *C. tropicalis* and *P. kudriavzevii* isolates, but additional multicenter comparison is mandatory to conclude on the possible superiority of AM3-MIC testing compared to the EUCAST method.

KEYWORDS FKS mutation, MIC distribution, anidulafungin, antifungal resistance, caspofungin, common yeast, micafungin, rare yeast, yeasts

Echinocandins inhibit cell wall synthesis by targeting the 1,3-β-d-glucan synthase encoded by *FKS* genes (1) and are the first-line recommended therapy of invasive candidiasis (2–4). Acquired resistance to echinocandins among yeasts remains rare (4–7). Some publications report a trend toward increasing the rate of echinocandin-resistant *Candida glabrata*, mostly in the USA but also recently in Germany (8–12), while others do not (13, 14). In any case, acquired echinocandin resistance has a major impact on patient management. It is therefore of utmost importance to reliably discriminate susceptible from resistant isolates in yeast species naturally susceptible to these drugs.

We previously showed that using caspofungin diluted in AM3 medium and a threshold of $\geq$ 0.5 mg/L is more stringent than RPMI to differentiate wild-type from non-wild-type isolates (i.e., with alteration in either Hot Spot (HS) 1 or 2 region of FKS protein) for *Candida albicans*, *Candida tropicalis* and *Pichia kudriavzevii* (synonym and current name for *Candida krusei*) (15–18). Between 2009 and 2014, we determined MIC of micafungin and caspofungin, using AM3 medium (AM3-MIC), and also MIC of anidulafungin using RPMI,

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according to the EUCAST procedure (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Files/EUCAST_E_Def_7.3.2_Yeast_testing_definitive_revised_2020.pdf). We describe here the echinocandins MIC distribution for clinical isolates belonging to 5 frequent and 16 rare pathogenic yeast species (19). The isolates, mainly recovered during prospective, multicentric surveillance programs in France, were all studied using the same procedure at the French National Reference Center for Invasive Mycoses & Antifungals (NRCMA). In addition, the suitability of our threshold (AM3-MIC ≥0.5 mg/L for caspofungin) was compared with that of the EUCAST breakpoints (BPs; anidulafungin MIC > 0.032 mg/L for *C. albicans* and >0.064 mg/L for *C. glabrata*, *C. tropicalis*, and *P. kudriavzevii* to detect isolates harboring FKS mutations (15–17, 20).

RESULTS

Echinocandins susceptibility distribution. A total of 2,787 clinical yeast isolates, mainly (88.1%) recovered from invasive human infections, were studied for their susceptibility to the three currently available echinocandins (Table 1; Table S1 in the supplemental material). The isolates belong to 21 different species (19 Ascomycetes corresponding to 11 genera, and 2 Basidiomycetes) with five species represented by more than 100 isolates (*C. albicans*, *C. glabrata*, *C. tropicalis*, *Candida parapsilosis*, and *P. kudriavzevii*).

The MIC90 for the three echinocandins was ≤ 0.06 mg/L for *C. albicans*, *C. dubliniensis*, and *C. tropicalis* while it was ≥ 0.5 mg/L for *C. parapsilosis*. *Candida parapsilosis sensu stricto* had higher MIC50 and MIC90 than *Candida metapsilosis* and *Candida orthopsilosis*, with *C. metapsilosis* exhibiting the lowest MIC values. *Candida glabrata* and *P. kudriavzevii* displayed caspofungin and anidulafungin MIC50 and MIC90 values higher than those obtained with *C. albicans*. Micafungin MICs were similar to those of *C. albicans* for *C. glabrata* but higher for *P. kudriavzevii*. *Clavispora lusitaniae* had MIC90 comparable to *P. kudriavzevii* for the three echinocandins (Table 1).

Among the rare species of Ascomycetes, *Candida inconspicua* and *Wickerhamomyces anomalus* behaved as *C. albicans* did while *Kluyveromyces marxianus* exhibited higher anidulafungin MIC90, and *Candida haemulonii* higher micafungin and anidulafungin MIC90. *Saccharomyces cerevisiae*, *Meyerozyma guilliermondii*, and *Yarrowia lipolytica* had MIC50 and MIC90 higher than those of *C. albicans* with anidulafungin MIC50 and MIC90 ≥ 1 mg/L for *M. guilliermondii*. Finally, *Saprochaete clavata*, *Magnusiomyces capitatus*, *Galactomyces candidus*, *Rhodotorula mucilaginosa*, and *Trichosporon asahii* had MIC90 ≥ 4 mg/L for the three echinocandins.

Strains ATCC22019 and ATCC6258 used as internal quality control exhibited stable anidulafungin, caspofungin, and micafungin MIC values over the study period. These MIC values were consistent with target values described by the EUCAST for anidulafungin (Fig. S1).

To compare our data with those recorded in the EUCAST database in terms of distribution and range of anidulafungin MICs, we used the online available EUCAST data (https://mic.eucast.org/Eucast2/SearchController/search.jsp?action=performSearch&BeginIndex=0&Micdif=mic&NumberIndex=50&Antib=716&Specium=-1). Both data sets (NRCMA and EUCAST) included similar number of isolates for the most common species. For *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *M. guilliermondii*, *C. glabrata*, and *P. kudriavzevii* isolates tested at the NRCMA, MIC distribution of anidulafungin was similar to that found online (Fig. 1A to 1F). Based on the EUCAST BP ($R > 0.03$ mg/L), 1.3% (15/1,198) of the NRCMA *C. albicans* isolates were resistant. Furthermore, 2.1% (5/238) of *C. tropicalis* isolates, 5.4% of *C. glabrata* (25/466), and 7.3% of *P. kudriavzevii* (10/137) were resistant to anidulafungin ($R > 0.06$ mg/L), while none of the *C. parapsilosis* was ($R > 4$ mg/L).

Local cutoff values were thus determined for isolates recovered in the YEASTS surveillance (14) program, i.e., without bias in isolates' selection and for species with more than 30 MIC values available (Table S2). Compared to the T-ECOFF (tentative epidemiological cutoff) values available on the anidulafungin rationale document (European Committee on Antimicrobial Susceptibility Testing, Anidulafungin: Rationale for the Clinical Breakpoints, version 3.0, 2020; http://www.eucast.org), the local cutoffs for anidulafungin

TABLE 1 Range of echinocandins MICs, MIC50, and MIC90 for the 2,787 yeast isolates received at the NRCMA between January 1 of 2009 and the December 31 of 2014, using EUCAST broth microdilution method

				Minimal inhibitory concentrations (MIC, mg/L)								
				Caspofungin[a]			Micafungin[a]			Anidulafungin		
Species	Synonym	*n*	Blood culture	MIC50	MIC90	Range	MIC50	MIC90	Range	MIC50	MIC90	Range
Candida albicans		1198	1068	0.03	0.06	≤0.007–≥4	0.03	0.03	≤0.007–2	≤0.007	≤0.007	≤0.007–0.5
Candida glabrata		466	398	0.06	0.125	0.03–≥4	0.03	0.03	≤0.007–2	0.03	0.06	≤0.007–2
Candida parapsilosis		282	261	0.25	1	0.03–2	0.5	0.5	0.125–2	1	1	0.125–2
Candida orthopsilosis		14	14	0.125	0.5	0.06–0.5	0.25	0.25	0.125–0.5	0.5	1	0.25–1
Candida metapsilosis		14	12	0.06	0.25	0.06–0.5	0.125	0.25	0.06–0.5	0.25	0.25	0.06–0.25
Candida tropicalis		238	214	0.03	0.06	≤0.007–≥4	0.03	0.03	≤0.007–2	0.015	0.03	≤0.007–1
Pichia kudriavzevii	*Candida krusei*	137	105	0.125	0.25	0.06–4	0.06	0.125	0.03–2	0.06	0.06	0.015–0.5
Clavispora lusitaniae	*Candida lusitaniae*	85	74	0,06	0,125	≤0.007–4	0.06	0.125	≤0.007–4	0.06	0.125	≤0.007–1
Saprochaete clavata	*Geotrichum clavatum*	72	61	≥4	≥4	1–≥4	≥4	≥4	0.25–≥4	4	4	1–4
Kluyveromyces marxianus	*Candida kefyr*	63	57	0.03	0.03	≤0.007–0.5	0.06	0.06	0.03–0.5	0.03	0.06	≤0.007–0.5
Candida dubliniensis		35	31	0.03	0.03	≤0.007–0.06	0.03	0.03	≤0.007–0.03	≤0.007	0.015	≤0.007–0.015
Trichosporon asahii		26	13	4	≥4	2–≥4	2	≥4	1–≥4	4	4	2–4
Meyerozyma guilliermondii	*Candida guilliermondii*	23	19	0,125	0,125	0.03–0.25	0.25	0.25	0.06–0.5	0.5	1	0.25–2
Magnusiomyces capitatus	*Geotrichum capitatum*	22	16	≥4	≥4	4–≥4	≥4	≥4	1–≥4	2	4	0.25–4
Galactomyces candidus	*Geotrichum candidum*	20	1	1	≥4	≤0.007–≥4	0.5	≥4	≤0.007–≥4	2	4	≤0.007–4
Candida inconspicua		20	19	0,06	0,06	0.03–0.125	0.03	0.03	≤0.007–0.03	≤0.007	0.03	≤0.007–0.03
Saccharomyces cerevisiae		19	44	0.125	0.25	0.125–0.25	0.125	0.25	0.06–0.25	0.06	0.25	0.03–0.5
Rhodotorula mucilaginosa		18	17	≥4	≥4	4–≥4	≥4	≥4	4–≥4	4	4	2–4
Candida haemulonii		13	8	0.03	0.06	0.03–0.25	0.06	0.125	0.06–0.125	0.06	0.125	0.03–0.125
Wickerhamomyces anomalus	*Candida pelliculosa*	12	11	0,06	0.06	0.03–0.06	0.03	0.03	≤0.007–0.03	0.015	0.015	≤0.007–0.03
Yarrowia lipolytica	*Candida lipolytica*	10	4	0.125	0.5	0.06–4	0.25	0.25	0.25–0.5	0.25	0.5	0.125–4

[a]Caspofungin and micafungin are diluted in AM3 medium.

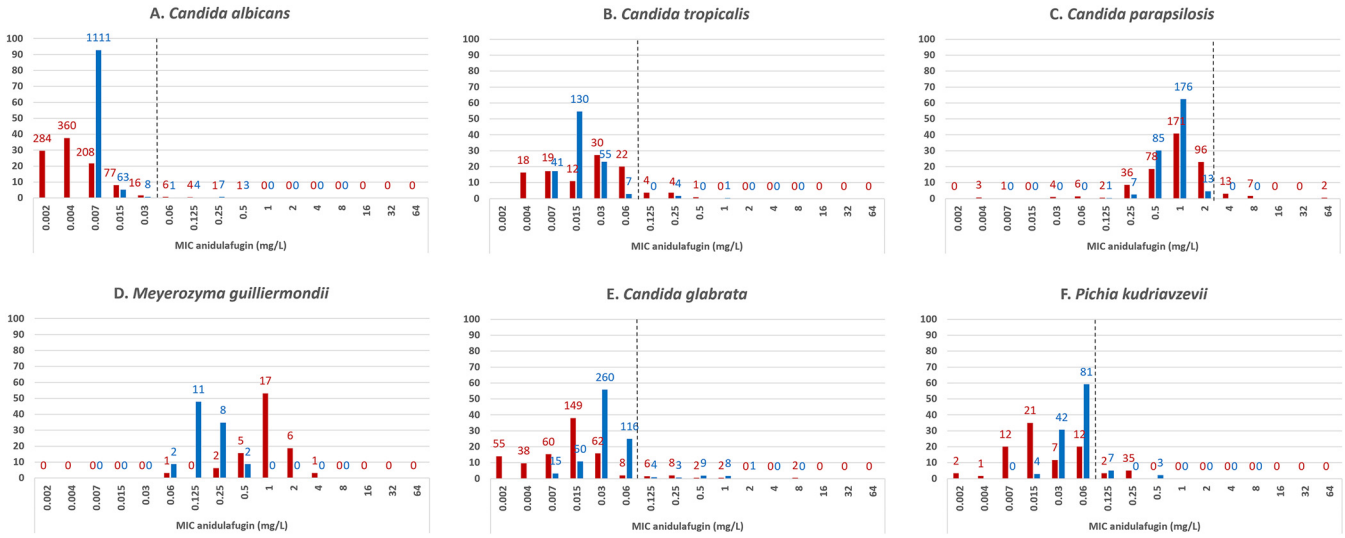

FIG 1 MIC distributions of anidulafungin for (A) *Candida albicans*, (B) *Candida tropicalis*, (C) *Candida parapsilosis*, (D) *Meyerozyma guilliermondii*, (E) *Candida glabrata*, (F) *Pichia kudriavzevii*. NRCMA data in blue, EUCAST data in red. The number of isolates is indicated for each MIC value. The dotted line corresponds to the EUCAST BP.

were higher (1 dilution) for *C. glabrata*, *C. tropicalis*, and (2 dilutions) *P. kudriavzevii*, and lower (1 dilution) for *C. albicans* and *C. parapsilosis*. ECOFF values and local cutoffs were not comparable for caspofungin and micafungin given that AM3 medium was used locally for dilution instead of RPMI. To assess whether the proportion of *C. albicans* and *C. glabrata* isolates with caspofungin AM3-MIC ≥ 0.5 mg/L increased over time, we analyzed the data according to the year of isolation up to 2020 since the YEASTS program is still ongoing (Fig. 2). We observed variations in the proportion of isolates with caspofungin AM3-MICs ≥ 0.5 mg/L (between 0 and 1.8% for *C. albicans*, and between 0 and 7.3% for *C. glabrata*), but no trends toward an increasing proportion of resistant isolates over time.

Analysis of FKS mutation. We sequenced *FKS* genes for the 64 isolates considered resistant to anidulafungin based on the EUCAST BP (15 *C. albicans*, 25 *C. glabrata*, 5 *C. tropicalis*, and 10 *P. kudriavzevii*) and those above the caspofungin threshold (20 *C. albicans*, 25 *C. glabrata*, 4 *C. tropicalis*, 5 *P. kudriavzevii*, and 1 *K. marxianus*). Of these 64 isolates, 46 were considered resistant using both criteria (15 *C. albicans*, 23 *C. glabrata*, 4 *C. tropicalis*, and 4 *P kudriavzevii*). Overall, 50/64 exhibited alterations in FKS protein: 20/20 *C. albicans*, 3/5 *C. tropicalis*, 21/27 *C. glabrata*, 5/11 *P. kudriavzevii*, and 1/1 *K. marxianus* (Table 2, Fig. 3).

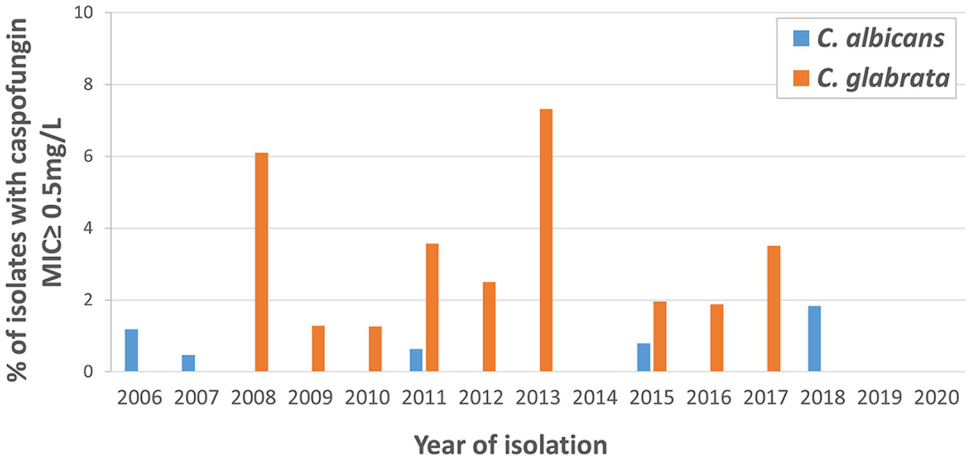

FIG 2 Percentages of *C. albicans* and *C. glabrata* isolates with caspofungin MIC ≥ 0.5 mg/L, according to the year of isolation. *Candida albicans* (blue) and *C. glabrata* (orange) isolates were recovered during the YEASTS surveillance program between 2006 and 2020. Linear trends curves were determined.

TABLE 2 Mutations in FKS for isolates of *C. albicans*, *C. glabrata*, *P. kudriavzevii*, *C. tropicalis*, and *K. marxianus* having caspofungin MIC ≥ 0.5 mg/L or resistant to anidulafungin according to the EUCAST BP

Species (nbr of isolates sequenced)	Nbr of isolates non-WT	Mutation	Localization	Range of MICs (mg/L)			Mutations described
				Caspo	Mica	Anidula	
Candida albicans (*n* = 20)	1	F641S	HS1	1	0.25	0.25	37
	9	S645P	HS1	4–>4	0.5 –>4	0.25–1	36
	8	S645S/P	HS1	0.5–2	0.25–1	0.015–1	36
	1	R647G	HS1	0.5	0.5	0.03	16
	1	R1361G	HS2	0.5	0.25	0.25	16
Candida glabrata (*n* = 27)	1	F625S	FKS1HS1	>4	0.5	2	11
	4	DelF659	FKS2HS1	4–>4	0.25–2	1–2	11
	1	F659S	FKS2HS1	0.5	0.06	0.5	43
	1	F659V	FKS2HS1	4	0.06	1	39
	7	S663P	FKS2HS1	1–>4	0.125–2	0.25–4	11
	1	S663F	FKS2HS1	1	0.25	1	43
	1	F659S +L664V	FKS2HS1	0.5	0.06	1	16
	1	S663P +M1439R	FKS2HS1 + FKS2 non-hotspot alteration	4	0.5	1	This study
	3	R1378S	FKS2HS2	1–4	0.06–0.125	0.5–1	53
	1	DelF659 + K335N	FKS2HS1 + FKS3 non-hotspot alteration	>4	1	2	This study
Pichia kudriavzevii (*n* = 11)	1	F655L	HS1	2	1	1	44
	2	S659F	HS1	4	2	0.5	38
	2	S659S/P	HS1	0.5–1	0.125	0.06–0.125	38
Candida tropicalis (*n* = 5)	2	S645P	HS1	4	0.5	0.5	54
	1	F641L + I1368S	HS1 +HS2	>4	2	2	This study
Kluyveromyces marxianus (*n* = 1)	1	F651F/S	HS1	0.5	0.5	1	This study

The 9 isolates considered resistant using EUCAST BP and "susceptible" using the NRCMA criteria (1 *C. tropicalis*, 2 *C. glabrata*, and 6 *P. kudriavzevii*) had a wild-type FKS sequence (Fig. 3B to D). Among the 8 isolates considered susceptible using EUCAST BP and "resistant" using the NRCMA criteria, 6 had FKS mutation (5 *C. albicans* and 1 *P. kudriavzevii*, Fig. 3A and D), and two had a wild-type FKS sequence (2 *C. glabrata* isolates, Fig. 3C).

Overall, 21 different mutations were identified, and the majority (17/21) were localized in the HS1 region. Five different mutations or combination of mutations were found for *C. albicans*, 10 for *C. glabrata*, 3 for *P. kudriavzevii*, 2 for *C. tropicalis*, and 1 for *K. marxianus* (Table 2).

For *C. albicans*, modification at S645 was the most prominent amino acid substitution (17/20) in homozygous and heterozygous forms (9/17 and 8/17, respectively; Table 2).

In *C. glabrata*, amino acid modification in S663 (S663P [*n* = 7]; S663F [*n* = 1]) and F659 (DelF659 [*n* = 4]; F659V [*n* = 1]; F659S [*n* = 1]) in HS1 of *FKS2* were the most frequent mutations. An unreported mutation localized upstream of the HS2 region of *FKS3* was also found. For three isolates, combination of mutations including one known mutation in HS1 of *FKS2* and another mutation localized downstream HS2 of *FKS2* or upstream HS1 of *FKS3* were found. The 4 isolates with deletion in position F659 had high MICs of the three echinocandins, whereas the five isolates with single or combined F659S or F659V substitution had low micafungin MIC (AM3-MIC = 0.06 mg/L). Four isolates with caspofungin AM3-MIC ≥ 0.5 mg/L had no mutation in HS1 nor HS2 for FKS1 2 and 3. Among those four isolates, two were resistant to anidulafungin (MIC = 0.125 mg/L) and two were susceptible (MIC ≤ 0.06 mg/L).

For *C. tropicalis*, two isolates had mutations S645P in HS1, one isolate had two mutations in HS1 and HS2, and for one isolate, amplification of the HS1 region was technically not possible. One isolate of *K. marxianus* with elevated MIC for the three echinocandins has an undescribed heterozygous mutation F651S.

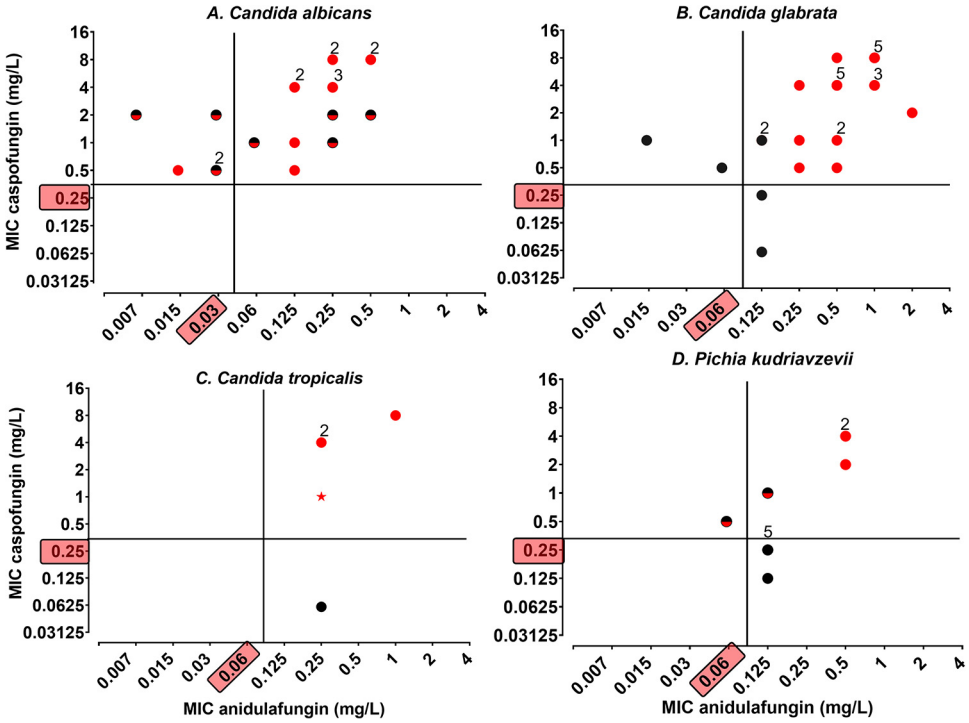

FIG 3 Schematic classification of isolates according to anidulafungin EUCAST BPs or NRCMA criteria (caspofungin MIC ≥ 0.5 mg/L), and FKS sequence. Isolates were defined as wild type (WT) when the FKS protein sequence was similar to that of the reference strain (black dot), and as non-wild-type (NWT) if it exhibited amino acid mutation in the HS1 or HS2 region (red dot for homozygous mutation; red and black dot for heterozygous mutation). The anidulafungin BP defined by the EUCAST AFST subcommittee and the CNRMA caspofungin threshold (MIC ≥ 0.5 mg/L) are highlighted in the *y* and *x* axis, respectively. The number of isolates is indicated above the dot when superior to 1. (A) Twenty *Candida albicans* isolates resistant to anidulafungin ($R > 0.03$ mg/L, $n = 15$) or caspofungin ($n = 20$). (B) Twenty-seven *Candida glabrata* isolates resistant to anidulafungin ($R > 0.06$ mg/L, $n = 25$) or caspofungin ($n = 25$). (C) Five *Candida tropicalis* isolates resistant to anidulafungin ($R > 0.06$ mg/L, $n = 5$) or caspofungin ($n = 4$). The star symbol corresponds to the isolate for which HS1 was not sequenced. (D) Eleven *Pichia kudriavzevii* isolates resistant to anidulafungin ($R > 0.06$ mg/L, $n = 10$) or caspofungin ($n = 5$).

Sensitivity (SE), specificity (SP), prevalence (P), positive and negative predictive values (PPV and NPV, respectively) were calculated on the subset of isolates that were sequenced to assess the efficacy of caspofungin or anidulafungin MIC determination and the EUCAST BPs/local thresholds in detecting isolates with FKS mutation (Table 3). The data set included 62 isolates belonging to species for which anidulafungin EUCAST BP were defined (*C. albicans*, *C. glabrata*, *C. tropicalis*, and *P. kudriavzevii*). PPV and NPV were 92% and 100% for caspofungin, and 80% and 25% for anidulafungin, respectively. The proportion of isolates with FKS mutation correctly identified (i.e., sensitivity) was 100% for caspofungin (NRCMA threshold) and 88% for anidulafungin (EUCAST BP), while the proportion of wild-type isolates correctly identified (i.e., specificity) was 69% for cas-

TABLE 3 Calculation of positive predictive value (PPV) and negative predictive value (NPV) of the caspofungin local threshold and anidulafungin BP for isolates of *C. albicans* ($n = 20$), *C. glabrata* ($n = 27$), *C. tropicalis* ($n = 4$), and *P. kudriavzevii* ($n = 11$)[a]

	FKS protein			
Categorization	**Non-WT**	**WT**	**PPV**	**NPV**
Caspofungin MIC ≥ 0.5 mg/L	TP = 49	FP = 4	92%	
Caspofungin MIC < 0.5 mg/L	FN = 0	TN = 9		100%
Anidulafungin R	TP = 43	FP = 11	80%	
Anidulafungin S	FN = 6	TN = 2		25%

[a]TP, true positives; FP, false positives; FN, false negatives; TN, true negatives. R and S, resistant or susceptible to anidulafungin according to the BP EUCAST; WT, wild-type FKS protein sequence.

pofungin (4 *C. glabrata* nonmutated isolates had caspofungin AM3-MIC $\geq$ 0.5 mg/L) and 15% for anidulafungin (11 isolates considered resistant to anidulafungin with no FKS mutation uncovered, including 4 *C. glabrata*, 6 *P. kudriavzevii*, and 1 *C. tropicalis*).

DISCUSSION

Thanks to the missions of the NRCMA, we have been able to generate over time antifungal susceptibility data concerning isolates of yeasts responsible for invasive infections. We here analyzed the echinocandins susceptibility of 2,787 isolates belonging to 21 yeast species, including the 5 most frequent ones in France, but also emerging and rare Ascomycetous and Basidiomycetous yeast species. The susceptibility profiles that we observed matched those usually reported for common and rare species (5, 19, 21–28).

We then assessed whether the EUCAST BPs for anidulafungin and our local threshold for caspofungin using our AM3-MIC testing lead to similar categorization of wild-type and mutated isolates. The anidulafungin EUCAST BPs were liable to categorize isolates without FKS mutation as resistant (1 *C. tropicalis*, 6 *P. kudriavzevii*, 4 *C. glabrata*) (12), but they also categorize isolates with mutation as susceptible (2 *C. albicans* and 1 *P. kudriavzevii*). However, for *C. glabrata* this threshold was also liable to categorize wild-type isolates as caspofungin resistant ($n = 4$), two of which were also considered as anidulafungin resistant according to the EUCAST BP. The absence of mutation associated with high MIC value for echinocandins is already described (12, 13, 29). One explanation is that these isolates corresponded to a mixture of susceptible and resistant isolates that we did not uncover despite testing single colonies. Another hypothesis is that the presence of a mutation localized in another part of the gene or in another gene was involved in the acquired resistance that would require whole genome sequencing to uncover. Furthermore, the concept of area of technical uncertainty (ATU) already described in the EUCAST standardized method, could be applied. In fact, *C. glabrata* isolates susceptible to anidulafungin and exhibiting caspofungin AM3-MIC of 0.5 or 1 mg/L do not harbor FKS protein alteration. Of note, given the mode of MIC distribution for anidulafungin observed in our center, an elevation of the anidulafungin BP value for *C. glabrata* by one dilution (MIC $>$ 0.125 mg/L) could be an option for us to correctly categorize the mutated isolates.

The EUCAST-AFST subcommittee has ruled out the use of caspofungin MICs to categorize isolates, because of inter and intralaboratory variations of MICs (8, 30, 31), and the same lack of laboratory-to-laboratory reproducibility was shown for CLSI testing (31). Thus, it considers that isolates of frequent species resistant to anidulafungin and micafungin should be considered resistant to caspofungin without further assessment (32). Nevertheless, we and another team already pointed out that determination of caspofungin MICs seems more reliable for detecting FKS mutated isolates than anidulafungin (16, 18, 33). We thus wonder whether reintroducing caspofungin in the panel of echinocandin drugs used to look for FKS-mutated isolates is not worth it.

Similarly, the EUCAST-AFST subcommittee does not recommend AM3 medium because of the possible variations related to manufacturers and even batches, the medium being more complex than RPMI (34). We did not observe variations in the caspofungin AM3-MIC for the quality control strains over the 6 years of the study (Fig. S1). We acknowledge that this lack of variation could rely on the single center evaluation. More specifically, plates and AM3 were purchased from unique manufacturers even if several batches were used over time, and inoculum determination and OD measurement were performed with the same readers, and by a single laboratory technician team. Finally, testing caspofungin in AM3 could modify the susceptibility profiles of the yeasts preventing its routine use, which was not the case.

To support our results, we looked for possible biases in our conclusions. We wondered whether the mutations observed were those usually described. Overall, of the 64 isolates sequenced, 50 harbored 21 different alterations in the FKS proteins. Resistance to echinocandins has been associated with amino acid substitutions in HS1 and/or HS2 regions on FKS protein for the most frequent species (*C. albicans*, *C. glabrata*, *C. tropicalis*, *P. kudriavzevii*) (8, 11, 20, 35–39) but also for less frequent species such as *C. lusitaniae*, *C. dubliniensis*, and *K.*

marxianus (20, 40–42). FKS alterations are most commonly substitutions in the HS1 region, but deletions and stop codons have also been reported in *C. glabrata* (8). In fact, FKS mutations are more frequently described for *C. glabrata* than for any other yeast species (5, 8, 16). Mutations found here for *C. albicans* and *C. glabrata* corresponded mostly to the mutations frequently described (43–45). We also found 2 previously unknown putative mutations localized near the HS1 or HS2 regions for *C. glabrata* isolates, 1 combination of mutation in HS1 and HS2 for *C. tropicalis*, and 1 unknown mutation in the HS1 region for *K. marxianus*. For *C. albicans*, S645P mutation was found in homozygous and heterozygous forms leading to higher caspofungin MICs for homozygous (AM3-MIC $>$ 2 mg/L) than heterozygous (AM3-MIC between 0.5 and 2 mg/L), suggesting the presence of wild-type and mutant enzymes as already reported (1). Even if MIC determination, DNA extraction, and FKS sequencing were performed on single colonies for isolates with heterozygous mutation, it does not exclude that a mixture of wild-type and resistant population was present in the original sample leading to low MIC value and detection of heterozygous form of enzyme. Influence of the amino-acid alteration on the MIC value has been previously reported with mutation in F641, S645, and R1361 associated with pronounced MIC elevations while other mutations such as R647 were associated with less elevated MIC values (46). This was not confirmed here. For *C. glabrata*, isolates with deletion in position F659 were "resistant" to three echinocandins, whereas isolates with F659S or F659V substitution have low AM3-MIC for micafungin (43). In the same way, isolates with a combination of mutations localized in positions 659 and 664 in the HS1 region had high MIC of caspofungin and anidulafungin but low MIC of micafungin (AM3-MIC = 0.06 mg/L). Among the 11 isolates of *P. kudriavzevii* studied, 5 had mutations in HS1 and also L701M heterozygous or homozygous mutation localized near the HS1 region. This mutation has already been described in echinocandin-susceptible isolates, and its implication in resistance is unclear (17, 44, 47). These results suggest that we did not introduce a bias due to a particular mutation when using our caspofungin threshold.

In conclusion, based on a large data set on isolates collected through prospective microbiological surveillance networks in France, we showed that detection of resistant isolates in common yeast species is not always trivial despite published BPs, and that our single center experience looks promising. Whether our results are convincing enough to trigger additional studies or whether alternative methods such as mass spectrometry using MALDI-TOF, or real-time PCR, can be more reliably used to detect resistant isolates remains to be determined (45, 48–51).

MATERIALS AND METHODS

Isolates. The French National Reference Center for Invasive Mycoses and Antifungals (NRCMA) provides expertise on isolates involved in invasive fungal infections from $>$200 hospitals in France. Between the January 1 of 2009 and the December 1 of 2014, all yeast isolates (n = 3,337) sent to the NRCMA were identified and tested for antifungal susceptibility. Isolates were sent mainly in the context of the epidemiological surveillance (72.5%, 2,418/3,337 including 1,963 isolates from the YEASTS program in the Paris area [6, 7]), or for specific expertise on species identification/typing or antifungal susceptibility testing (27.5%, 918/3,337). Isolates of *Cryptococcus neoformans* and *Cryptococcus gattii* species complex were excluded from this study. Species with 10 or more isolates were analyzed in the present study (n = 2,787). Isolates were identified at the species levels as previously described (52).

The dual source of isolates prevented calculation of echinocandin resistance incidence. Nevertheless, we used the exhaustive YEASTS surveillance program on candidemia in the Paris area to determine the percentage of *C. albicans* and *C. glabrata* isolates with caspofungin AM3-MIC $\geq$ 0.5 mg/L according to the year of isolation between 2006 and 2020.

Antifungal susceptibility. MICs were determined for anidulafungin by using the standardized broth microdilution method from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (sterile tissue culture plates, 96-well flat bottom in clear polystyrene, TPP Techno Plastic Products AG, Switzerland, Reference 92096). For caspofungin and micafungin, AM3 medium (BD Difco, USA, Reference 224320) was used for dilution (15–18). All MICs were determined on the same day using the same inoculum. Quality control strains (ATCC22019, ATCC6258) were included in each set. The concentrations corresponding to the MIC that inhibited 50% (MIC50) and 90% (MIC90) of the isolates were determined for species with 10 or more isolates studied (Table 1 MICs). ECOFFs (epidemiological cutoff values) are defined by EUCAST as "the highest MIC for organisms devoid of phenotypically detectable acquired resistance mechanisms" and correspond to the upper end of the wild-type MIC distribution. Given that our data come from a unique center, we could only determine local cutoff values. We thus calculate 99.9% local cutoff values for anidulafungin, micafungin, and caspofungin, for species considered as susceptible to echinocandins (excluding *S. clavata*) with 30 or more isolates tested, following the

TABLE 4 Primers used for amplification and sequencing of HS1 and HS2 regions of *FKS* genes

Species	Region	Primer	Sequence 5′ 3′	Ref
Candida albicans	HS1	GSC1f	GAAATCGGCATATGCTGTGTC	36
		GSC1r	AATGAACGACCAATGGAGAAG	
	HS2	CAS2f	ACCACCAAGATTGGTGCTG	17
		CAS2r	TATCTAGCACCACCAACGG	
Pichia kudriavzevii	HS1	CKS1f	ACTGCATCGTTTGCTCCTCT	17
		CKS1r	GAACATGATCAATTGCCAAC	
	HS2	CKS2f	CCGGTATGGGAGAACAAATG	
		CKS2r	CACCACCAATGGAAACATCA	
Candida tropicalis	HS1	CTS1-1f	ATGGTTCAGTATAGGTGGATG	17
		CTS1-1r	AAGGAACGACCAATGGAGAAG	
	HS2	CTS1-2f	ACTACCAAGATTGGTGCTG	
		CTS1-2r	TATCTAGCACCACCAACAG	
Candida glabrata	FKS2-HS1	FKS2F	GGCCACTGTTTTATTCTTCTCG	35
		FKS2R	GTAAATGTTCTCTGTACATGGA	
	FKS2-HS2	CG2f	ACAACTAAGATTGGTGCAG	54
		CG2r	TAACGAGCACCACCCACA	
	FKS1-HS1	FKS1f	GTTGCAGTCGCTACATTGCTA	35
		FKS1r	TAGCGTTCCAGACTTGGGAA	
	FKS1-HS2	FKS1HS2f	GGTATTTCAAAGGCTCAAAAGGG	39
		FKS1HS2r	ATGGAGAGAACAGCAGGGCG	
	FKS3-HS1	FKS3f	TGGAGCCCAGCACTTAACAA	35
		FKS3r	GTCCATCTCGGATGTTGCTA	
	FKS3-HS2	CG3-HS2f	TTATGCAGAGGAACCTGCTC	54
		CG3-HS2r	GTGCCATCGACAGTAAGTGA	
Kluyveromyces marxianus	HS1	CKHS1f	GGTGGTTTATTCACTTCCTACA	42
		CKHS1r	GCGTAGCCAAAGATTGAGCA	
	HS2	CKHS2f	AAGATTGGTGCYGGTATGGG	
		CKHS2r	RGTDGCAAAACCTCTAGCAGT	

EUCAST SOP10 recommendation (MIC distributions and epidemiological cutoff value [ECOFF] setting, EUCAST SOP 10.0, 2017. http://www.eucast.org) and using the ECOFFfinder program v2.0. To avoid bias of selection, isolates not recovered during the YEASTS program (active surveillance program of fungemia in the Paris area [19]) were excluded. The clinical BPs or T-ECOFF values determined by EUCAST for some species and some antifungal agents were used to calculate percentage of resistant (R) isolates (https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/AFST/Clinical_breakpoints/AFST_BP_v10.0_200204_updatd_links_200924.pdf; https://mic.eucast.org/Eucast2/SearchController/).

FKS sequencing. According to previous studies, Hot Spot (HS) 1 and 2 regions of the *FKS* gene were sequenced for *C. albicans*, *C. glabrata*, *C. tropicalis*, *P. kudriavzevii*, and *K. marxianus* isolates having AM3-MIC caspofungin ≥ 0.5 mg/L, with primers listed in Table 4. Of note, for *C. glabrata* HS1 and HS2 regions were sequenced for *FKS1*, *FKS2*, and *FKS3* genes. Reference strains (*C. albicans* CBS 562, *C. glabrata* ATCC 2001, *C. tropicalis* ATCC 750, *P. kudriavzevii* ATCC 6258, *K. marxianus* CBS 712) were used as positive control for PCR amplification and as wild-type reference for *FKS* protein sequences. For *K. marxianus*, numbering was based on the protein sequence of the 1-3beta glucan synthase (GenBank accession number BAO40851.1). For isolates resistant to anidulafungin and/or with AM3-MIC caspofungin ≥ 0.5 mg/L and having a wild-type sequence, at least five colonies were checked for MIC and FKS sequences. For the 12 isolates with heterozygous mutation in FKS (8 *C. albicans*, 2 *P. kudriavzevii*, and 1 *K. marxianus*), two single colonies were isolated. For these single colonies, DNA extraction, FKS sequencing of the HS regions, and MIC determination were performed. Among the 12 isolates analyzed, two *P. kudriavzevii* (CNRMA13.91, CNRMA12.1267) were identified as a possible mixture of wild-type and mutated isolates, but only mutated isolates were recovered, and one *C. albicans* (CNRMA9.37) was identified as a mixture of wild type and resistant isolates with wild-type FKS and mutated (heterozygous) FKS, respectively.

Positive and negative predictive values. Positive and negative predictive values (PPV and NPV, respectively) correspond to the proportion of positive and negative results in a test that are true positive and true negative results, respectively. In the present study, PPV indicates the proportion of non-wild-type isolates for isolates resistant to anidulafungin (based on EUCAST BPs) or for isolates with caspofungin AM3-MIC ≥ 0.5 mg/L. The ideal value of PPV and NPV is 1 (100%).

Sensitivity (SE), specificity (SP), and prevalence (P) were determined to calculate PPV and NPV, using the number of wild-type and non-wild-type isolates according to the MIC value for caspofungin or anidulafungin. The sensitivity (SE) measures the proportion of positives correctly identified (i.e., isolates with FKS mutation resistant to anidulafungin or with caspofungin AM3-MIC ≥ 0.5 mg/L). The specificity

(SP) measures the proportion of negatives correctly identified (i.e., wild-type isolates susceptible to anidulafungin or with caspofungin AM3-MIC < 0.5 mg/L). SE, SP, and P were calculated as follows:

$$\mathrm{SE} = \text{number of true positives}/(\text{number of true positives} + \text{number of false negatives})$$

$$\mathrm{SP} = \text{number of true negatives}/(\text{number of true negatives} + \text{number of false positives})$$

$$\mathrm{P} = (\text{number of true positives} + \text{number of false negatives})/\text{total number of populations}$$

PPV and NPV were calculated as follows:

$$\mathrm{PPV} = \mathrm{SExP}/[\mathrm{SE} * \mathrm{P} + (1 - \mathrm{P})\mathrm{x}(1 - \mathrm{SP})]$$

$$\mathrm{NPV} = \mathrm{SPx}(1 - \mathrm{P})/[(\mathrm{SPx}(1 - \mathrm{P}) + \mathrm{Px}(1 - \mathrm{SE})]$$

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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