AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

SUSCEPTIBILITY

EUCAST Reference Testing of Rezafungin Susceptibility and Impact of Choice of Plastic Plates

Maiken Cavling Arendrup,[a,b,c] Karin Meinike Jørgensen,[a] Rasmus Krøger Hare,[a] Manuel Cuenca-Estrella,[d] Oscar Zaragoza[d]

[a]Unit of Mycology, Statens Serum Institut, Copenhagen, Denmark
[b]Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark
[c]Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark
[d]Mycology Reference Laboratory, National Centre for Microbiology, Instituto de Salud Carlos III, Madrid, Spain

ABSTRACT Rezafungin is a new long-acting echinocandin currently in phase 3 development. Epidemiological cutoff values are necessary for breakpoint setting but have not been established due to unexplained interlaboratory MIC variations observed in a prior multicenter study. Here we investigated if the choice of microtiter plates affected the variability when anidulafungin was included as a comparator. Testing by the EUCAST E.Def 7.3.1 reference method using tissue and cell culture-treated polystyrene plates (TC plates) and untreated polystyrene plates (UT plates) from four manufacturers was performed. Six control strains (*Candida albicans*, $n = 3$; *C. krusei*, $n = 2$; *C. parapsilosis*, $n = 1$) were tested (520 MICs). Subsequently, 5 or 6 wild-type isolates and 4 or 5 *fks* mutants of *C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* (wild type only), and *C. tropicalis* were tested (930 MICs). For each strain-plate combination, ≥98% of the repetitive MICs were within 3 dilutions. The rezafungin modal MICs for the collated *C. albicans* control strain distributions were 0.016 mg/liter across TC plates but 0.03 mg/liter across UT plates, whereas they were 0.004 mg/liter and 0.016 mg/liter, respectively, for anidulafungin. The difference was most pronounced with Falcon plates and was not observed for *C. krusei* and *C. parapsilosis*. Eleven rezafungin MICs for mutants overlapped with the MICs for wild-type isolates (TC plates, $n = 4$; UT plates, $n = 7$). For anidulafungin, five overlaps (all UT plates) were observed. Most overlaps (rezafungin, $n = 5$; anidulafungin, $n = 3$) were caused by *fks* mutants of *C. tropicalis* (Fks1, F650F/L) and *C. glabrata* (Fks2. D666Y; rezafungin, $n = 2$; anidulafungin, $n = 1$). Interlaboratory variation was low. The use of TC plates resulted in lower MICs, particularly for *C. albicans* and Falcon plates, ad this was more often the case for anidulafungin than for rezafungin. Adoption of TC plates for EUCAST antifungal susceptibility testing would improve interlaboratory reproducibility and the separation of non-wild-type and wild-type strains.

KEYWORDS *Candida*, antifungal susceptibility testing, broth microdilution, echinocandins

Rezafungin is a new long-acting echinocandin currently in phase 3 development for the treatment of candidemia and candidiasis and for the prevention of infections caused by *Candida*, *Aspergillus*, and *Pneumocystis* spp. in bone marrow transplant patients (1–4). A recently published multicenter study revealed an unexpected interlaboratory variation among rezafungin EUCAST MICs generated in the four European mycology reference laboratories (5). This variation was particularly prominent for the species with the lowest MIC, *Candida albicans*. The source of the variation was not clear. The four laboratories all followed the ISO procedure for the preparation of drug dilutions for plate production, thus avoiding serial dilution and any potential variation associated therewith. Three of the four laboratories shared the same lot of pure

Citation Arendrup MC, Jørgensen KM, Hare RK, Cuenca-Estrella M, Zaragoza O. 2019. EUCAST reference testing of rezafungin susceptibility and impact of choice of plastic plates. Antimicrob Agents Chemother 63:e00659-19. https://doi.org/10.1128/AAC.00659-19.

Address correspondence to Maiken Cavling Arendrup, maca@ssi.dk.

Received 28 March 2019
Returned for modification 11 May 2019
Accepted 30 June 2019
Accepted manuscript posted online 8 July 2019
Published 23 August 2019

substance. The wavelength range for plate reading used by the laboratories generating high MICs overlapped the wavelength range used by the laboratories generating low MICs. Finally, all four centers used treated (tissue or cell culture-treated) polystyrene plates (TC plates) to avoid the variation that has previously been associated with echinocandin MIC testing across TC plates and untreated polystyrene plates (UT plates) (6). One consideration was that the pure substance was shipped without temperature control. Extreme temperatures during transport may potentially influence the biological potency of the drug powder. However, prior studies had demonstrated the high stability of rezafungin, including <2% degradation when lyophilized powder was stored at 40°C for 9 months (2). This suggests that shipping conditions were a less likely cause of the observed interlaboratory variation. The remaining potential source of variation was that the polystyrene plates and utensils for plate preparation were derived from different manufacturers. Hence, the possibility of variability in drug binding to the plastics could not be excluded, despite the fact that all centers used treated rather than untreated plates. The aim of this study was to thoroughly investigate and compare the rezafungin EUCAST MIC variability across eight different polystyrene plates, consisting of two different types of microtiter plates from each of four different manufacturers, against control strains and clinical *Candida* isolates with and without *fks* hot spot mutations. This selection included strains with a broad MIC range, as binding to plastic may affect such isolates differentially. Furthermore, this study expanded beyond the earlier multicenter study with the inclusion of anidulafungin as an in-class comparator agent. The motivation for the study was to determine whether further standardization of EUCAST rezafungin susceptibility testing is necessary in order to allow future rezafungin clinical breakpoint setting.

RESULTS

Repetitive testing of QC strains. Six control strains (three *C. albicans* strains, two *C. krusei* strains, and one *C. parapsilosis* strain) were tested for their susceptibility to rezafungin and anidulafungin 2 to 13 times on each of the eight different plate types, yielding a total of 520 MICs (Table 1). In 14 (28%) plate-control strain combination cases, the repetitive rezafungin MICs fell at the same MIC, in 27 (54%) cases the MICs spanned 2 dilutions, and in 9 (18%) cases the MICs spanned 3 dilutions. For anidulafungin, in comparison, the MICs of repetitive testing spanned 1, 2, and 3 dilutions in 21 (42%), 24 (48%), and 4 (8%) cases, respectively, and on one occasion (2%) (the Greiner TC plate and *C. krusei* ATCC 6258), the MIC distribution spanned 4 dilutions. These data suggest an acceptable intra-assay performance for all of the plates for both compounds.

The MICs were, overall, lower when generated using treated plates than when generated using untreated plates. Thus, the modal MICs for rezafungin and the collated *C. albicans* quality control (QC) strain distributions were 0.016 mg/liter across the treated plates but 0.03 mg/liter across the untreated plates (Table 1). This trend was more pronounced for anidulafungin, with modal MICs being 0.004 mg/liter and 0.016 mg/liter for the treated and untreated plates, respectively. This discrepancy was not observed for the compiled data set for the two *C. krusei* QC strains (modal MICs for rezafungin of 0.06 mg/liter for both plate types and modal MICs for anidulafungin of 0.03 mg/liter for both plate types) and also was not observed for the *C. parapsilosis* control strain for the plates tested in laboratory 1.

Comparing TC versus UT plates from each of the manufacturers pairwise, the compiled rezafungin MICs for the three *C. albicans* QC strains for the Falcon plates differed, with a 0.008- to 0.016-mg/liter range being found for the treated Falcon plates but a 0.016- to 0.125-mg/liter range being found for the untreated plates, whereas the MICs for the TC plates versus the UT plates from the other brands overlapped. In contrast, the anidulafungin MICs were lower for treated plates than for untreated plates for all four brands.

MICs for clinical wild-type isolates. The rezafungin MICs were the lowest for *C. albicans* and the highest for *C. parapsilosis*, as previously shown (4, 5) (Table 2; see also Table S1 in the supplemental material). *C. tropicalis* was identified to be the second

TABLE 1 Rezafungin and anidulafungin MIC results for repetitive testing of six QC strains[a]

		No. of isolates with the indicated MIC (mg/liter)																		
		Rezafungin									Anidulafungin									
QC strain and plate type	Lab no.	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	Total
C. albicans ATCC 64548																					
Nunc TC	1		**3**	2							**5**										5
Greiner TC	1		**4**	1							**4**	1									5
Greiner TC	2	1	1									2									2
Costar TC	2			**2**							1	1									2
Falcon TC	2	**2**									1	1									2
Nunc UT	1		**4**	1								**4**	1								5
Greiner UT	1		**3**	2								**2**	**2**	1							5
Costar UT	1			**5**									2	**3**							5
Costar UT	2		**2**										**2**								2
Falcon UT	2		1	1								**2**									2
C. albicans ATCC 64550																					
Nunc TC	1		**3**	2							**5**										5
Greiner TC	1		1	**3**	1						1	**3**	1								5
Greiner TC	2		1	1								**2**									2
Costar TC	2		1	1							1	1									2
Falcon TC	2	1	1									**2**									2
Nunc UT	1		1	**4**							1	**4**									5
Greiner UT	1			**5**								2	**3**								5
Costar UT	1			**4**		1							**4**	1							5
Costar UT	2		1		1								**2**								2
Falcon UT	2			1	1							1	1								2
C. albicans F8555																					
Nunc TC	1		**3**	2							**5**										5
Greiner TC	1		1	**2**	**2**							**4**	1								5
Greiner TC	2		1	1								1	1								2
Costar TC	2		1	1							1		1								2
Falcon TC	2	1	1								1	1									2
Nunc UT	1			**4**	1							**5**									5
Greiner UT	1			1	**4**								**5**								5
Costar UT	1				**4**	1							2	**3**							5
Costar UT	2			1	1									**2**							2
Falcon UT	2					**2**							**2**								2
C. albicans QC strains combined																					
TC plates		5	**22**	18	3						**25**	19	4								48
UT plates			12	**29**	12	4					1	20	**26**	10							57
C. krusei ATCC 6258																					
Nunc TC	1				**4**	1								**4**	1						5
Greiner TC	1				1	**3**	1							**3**	2						5
Greiner TC	2				**8**	5						1		**8**	4						13
Costar TC	2			4	**8**	1							4	**8**	1						13
Falcon TC	2			1	**12**								2	**11**							13
Nunc UT	1				**5**									**3**	2						5
Greiner UT	1				**4**	1									**5**						5
Costar UT	1			**2**	**2**	1									**3**	2					5
Costar UT	2		4	**7**	2									**9**	4						13
Falcon UT	2			**10**	3									**10**	3						13
C. krusei CL3403																					
Nunc TC	1				**4**	1								**5**							5
Greiner TC	1					**5**								1	**4**						5
Greiner TC	2				**2**										**2**						2
Costar TC	2			1	1									**2**							2
Falcon TC	2				**2**									1	1						2

(Continued on next page)

TABLE 1 (Continued)

QC strain and plate type	Lab no.	No. of isolates with the indicated MIC (mg/liter)																			
		Rezafungin									Anidulafungin										
		0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	Total
Nunc UT	1				**5**									**3**	2						5
Greiner UT	1				**5**										**5**						5
Costar UT	1			1	**2**	**2**										**5**					5
Costar UT	2			**2**										**2**							2
Falcon UT	2			**2**										**2**							2
C. krusei QC strains combined																					
TC plates				6	**42**	16	1					1	6	**43**	15						65
UT plates			4	24	**28**	4								**29**	24	7					60
C. parapsilosis ATCC 22019																					
Nunc TC	1								2	**4**									**6**		6
Greiner TC	1								3	**3**									**5**	1	6
Nunc UT	1								**4**	2									**5**	1	6
Greiner UT	1								1	**5**									1	**5**	6
Costar UT	1								1	**5**										**6**	6

[a]The table is organized according to QC strain and the type of plate (tissue and cell culture-treated [TC] versus untreated [UT] plates). The most common MICs are highlighted in bold.

least susceptible species by the Nunc TC, Costar UT, and Falcon UT plates and, in general, was the species for which the MIC distributions were the widest across both the different brands and the different laboratories. The modal MICs were similar for rezafungin when the collated distributions for TC plates versus UT plates were compared, although for *C. albicans* ($P = 0.04$ for treated versus untreated plates) and *C. glabrata* ($P < 0.001$), the MIC values for wild-type strains were significantly lower in treated plates (Table S1). For Falcon plates specifically, TC plates yielded lower rezaf-

TABLE 2 Rezafungin and anidulafungin MICs for *Candida* wild-type isolates, by plate type and laboratory

Antifungal, plate	Lab no.	Median (range) MIC (mg/liter)				
		C. albicans	*C. glabrata*	*C. krusei*	*C. parapsilosis*	*C. tropicalis*
Rezafungin						
Nunc TC	1	0.03 (0.03)	0.06 (0.06–0.125)	0.06 (0.03–0.125)	2 (1–2)	0.125 (0.06–0.125)
Greiner TC	1	0.03 (0.016–0.03)	0.09 (0.06–0.125)	0.125 (0.06–0.125)	2 (2)	0.125 (0.03–0.25)
Greiner TC	2	0.03 (0.03)	0.125 (0.06–0.125)	0.125 (0.016–0.25)	1.5 (1–2)	0.06 (0.016–0.06)
Costar TC	2	0.06 (0.03–0.06)	0.125 (0.125)	0.06 (0.03–0.06)	1.5 (1–2)	0.125 (0.06–0.125)
Falcon TC	2	0.016 (0.016)	0.03 (0.03–0.06)	0.06 (0.008–0.06)	1.5 (1–2)	0.03 (0.008–0.03)
Nunc UT	1	0.016 (0.016–0.03)	0.06 (0.03–0.06)	0.06 (0.03–0.06)	1 (1–2)	0.03 (0.03–0.125)
Greiner UT	1	0.016 (0.016–0.03)	0.05 (0.03–0.06)	0.06 (0.03–0.06)	2 (1–2)	0.06 (0.016–0.125)
Costar UT	1	0.03 (0.03–0.125)	0.06 (0.06–0.125)	0.06 (0.06–0.125)	2 (1–2)	0.125 (0.06–0.25)
Costar UT	2	0.06 (0.03–0.125)	0.06 (0.06)	0.06 (0.03–0.06)	1.5 (1–2)	0.125 (0.06–0.25)
Falcon UT	2	0.06 (0.03–0.125)	0.06 (0.06)	0.03 (0.03)	1 (0.5–2)	0.125 (0.03–0.25)
Anidulafungin						
Nunc TC	1	0.008 (0.004–0.008)	0.03 (0.016–0.03)	0.03 (0.016–0.03)	1 (0.5–1)	0.016 (0.016)
Greiner TC	1	0.008 (0.004–0.008)	0.03 (0.03–0.06)	0.03 (0.03–0.06)	1 (0.5–2)	0.016 (0.016–0.03)
Greiner TC	2	0.008 (0.008)	0.03 (0.03–0.06)	0.06 (0.004–0.125)	2 (1–2)	0.016 (0.002–0.03)
Costar TC	2	0.004 (0.004–0.008)	0.03 (0.03)	0.03 (0.004–0.06)	1.5 (0.5–2)	0.016 (0.008–0.016)
Falcon TC	2	0.004 (0.002–0.004)	0.016 (0.016–0.03)	0.03 (0.004–0.06)	2 (1–4)	0.016 (0.004–0.016)
Nunc UT	1	0.008 (0.008)	0.03 (0.03–0.06)	0.06 (0.03–0.06)	1 (1–2)	0.03 (0.008–0.06)
Greiner UT	1	0.016 (0.008–0.016)	0.06 (0.03–0.06)	0.06 (0.03–0.06)	2 (1–2)	0.03 (0.016–0.125)
Costar UT	1	0.016 (0.016–0.03)	0.125 (0.06–0.125)	0.125 (0.06–0.125)	2 (1–2)	0.06 (0.03–0.125)
Costar UT	2	0.016 (0.016–0.03)	0.06 (0.06)	0.06 (0.016–0.06)	4 (1–4)	0.06 (0.03–0.06)
Falcon UT	2	0.016 (0.008–0.016)	0.06 (0.06)	0.06 (0.016–0.06)	2 (1–2)	0.06 (0.03–0.06)

TABLE 3 Rezafungin and anidulafungin MIC ranges for *Candida* non-wild-type isolates and number of mutant isolates for which the MIC range overlapped the MIC range for the corresponding wild-type isolates, by plate type and laboratory[a]

Antifungal and plate	Lab no.	*C. albicans* MIC range (mg/liter)	*C. albicans* No. of overlaps	*C. glabrata* MIC range (mg/liter)	*C. glabrata* No. of overlaps	*C. krusei* MIC range (mg/liter)	*C. krusei* No. of overlaps	*C. tropicalis* MIC range (mg/liter)	*C. tropicalis* No. of overlaps
Rezafungin									
Nunc TC	1	0.125–1	0	0.125–2	1 (D666Y)	0.25–8	0	0.125–1	1 (F650F/L)
Greiner TC	1	0.06–0.5	0	0.25–2	0	0.25–8	0	**0.125–1**	**1 (F650F/L)**
Greiner TC	2	0.25–1	0	0.5–4	0	0.25–2	1 (P663Q)	0.25–1	0
Costar TC	2	0.25–1	0	0.25–4	0	0.25–2	0	0.25–1	0
Falcon TC	2	0.06–1	0	0.25–4	0	0.25–2	0	0.25–1	0
Nunc UT	1	0.125–1	0	0.125–2	0	0.25–8	0	0.125–1	1 (F650F/L)
Greiner UT	1	0.125–1	0	0.125–2	0	0.25–8	0	0.125–1	1 (F650F/L)
Costar UT	1	**0.06–1**	**1 (R647G)**	0.125–4	1 (D666Y)	0.25–8	0	**0.06–1**	**1 (F650F/L)**
Costar UT	2	0.125–1	1 (R1361H)	0.25–4	0	0.25–2	0	0.5–1	0
Falcon UT	2	0.125–1	1 (R1361H)	0.25–4	0	0.125–2	0	0.5–1	0
Anidulafungin									
Nunc TC	1	0.016–0.5	0	0.06–2	0	0.125–4	0	0.03–0.5	0
Greiner TC	1	0.016–0.5	0	0.125–2	0	0.25–8	0	0.06–0.5	0
Greiner TC	2	0.03–1	0	0.125–2	0	0.25–4	0	0.125–0.5	0
Costar TC	2	0.03–0.25	0	0.125–2	0	0.125–2	0	0.125–0.25	0
Falcon TC	2	0.03–2	0	0.25–2	0	0.25–4	0	0.125–0.5	0
Nunc UT	1	0.03–1	0	0.125–2	0	0.25–8	0	0.06–0.5	1 (F650F/L)
Greiner UT	1	0.06–1	0	0.125–2	0	0.25–8	0	**0.06–1**	**1 (F650F/L)**
Costar UT	1	0.06–2	0	0.125–4	2 (F659S, D666Y)	0.25–8	0	0.125–1	1 (F650F/L)
Costar UT	2	0.125–2	0	0.25–4	0	0.25–4	0	0.125–0.5	0
Falcon UT	2	0.125–2	0	0.25–4	0	0.25–4	0	0.25–0.5	0

[a]Number of overlaps indicates the number of mutant isolates for which the MIC range overlapped the MIC range for the corresponding wild-type isolate by plate type and laboratory. Specific amino acid alterations are indicated in parentheses. Bold and underlined font is used for highlighting major overlaps for which the overlap between the MICs for the mutant and wild-type isolates span several 2-fold dilutions. In contrast, normal font is used for minor overlaps, defined as MICs for non-wild-type isolates overlapping only at the highest MIC value observed for wild-type isolates.

ungin MICs than UT plates: 0.016 mg/liter (range, 0.016 mg/liter) versus 0.06 mg/liter (range, 0.03 to 0.125 mg/liter) ($P < 0.001$) for *C. albicans* and 0.03 mg/liter (range, 0.008 to 0.03 mg/liter) versus 0.125 mg/liter (range, 0.03 to 0.25 mg/liter) ($P = 0.007$) for *C. tropicalis* (Table S2e). In comparison, for anidulafungin, TC plates, in general, yielded modal MICs and ranges for all plate brands and species except *C. parapsilosis* lower than the modal MICs and ranges for UT plates and, again most profoundly, for the Falcon plates (Table 2 and Table S1). Finally, the interclass correlation of the MICs for wild-type isolates tested using Greiner TC and Corning UT plates in both laboratories was determined. The interclass correlation coefficient value was very high (0.93 [95% confidence interval, 0.91 to 0.95]), suggesting that the differences observed among plates in this study were not due to interlaboratory variation.

MICs for clinical Fks mutant isolates. The MICs of both rezafungin and anidulafungin were higher for the mutant isolates than for the wild-type isolates (Table 3). For rezafungin, the MIC range for the mutants overlapped the MIC range for the wild-type isolates on 11 occasions, including on 4 occasions for TC plates and on 7 occasions for UT plates. For anidulafungin, MIC overlaps were observed on 5 occasions, and all involved UT plates. Most overlaps ($n = 5$ for rezafungin, $n = 3$ for anidulafungin) were caused by a *C. tropicalis* strain harboring an F650F/L alteration, followed by a *C. glabrata* strain harboring the D666Y alteration ($n = 2$ for rezafungin, $n = 1$ for anidulafungin). On 12 occasions, the MICs for the mutant isolates were at the highest MIC of the range for the wild-type isolates. Finally, there were no statistically significant differences between the MIC values obtained in different plates for the mutant isolates ($P > 0.1$).

DISCUSSION

This study extends the findings of a prior rezafungin multicenter study in which notable differences, particularly for the most susceptible *C. albicans* species, were found

across four experienced EUCAST network laboratories (5). Prior studies have found that the most susceptible species are more prone to MIC variation (7, 8). For example, the caspofungin modal MIC variability was greater for *C. albicans* than for *C. glabrata* and *C. parapsilosis*, spanning 6, 5, and 4 2-fold dilutions, respectively, for CLSI MICs in a multicenter study (8). Therefore, it may not be surprising that the variation was greater for anidulafungin than for rezafungin, as anidulafungin MICs, in general, are lower than rezafungin MICs for all species except *C. parapsilosis*.

The repeated testing of control strains demonstrated that, for each plate type and lab, intralaboratory variability was acceptable, with more than 80% of the MICs falling within 2 dilutions. This suggests that factors other than human errors accounted for the interlaboratory variability. When compiling the data across the two plate types for the *C. albicans* control strains, the modal MIC was 1 dilution lower for rezafungin and 2 dilutions lower for anidulafungin when using TC plates rather than UT plates.

In a recent multicenter study, rezafungin MICs were the lowest in the laboratory that used Falcon tissue culture-treated plates and the highest in the laboratory that used Costar tissue culture-treated plates (modal MICs for *C. albicans*, 0.008 and 0.03 mg/liter, respectively), a finding that we reproduced here for the control strains as well as for the clinical wild-type isolates (0.016 and 0.03 mg/liter, respectively). These observations may help explain the notable variability found in that study. Taken together, these data suggest a plate-specific difference in the performance of MIC testing, which, although the difference was less pronounced for rezafungin than for anidulafungin, may complicate standardization of EUCAST reference testing unless the type and brand of microtiter plates are chosen carefully.

When the performance with respect to differentiation between wild-type and Fks mutant isolates was investigated, fewer overlaps were observed when TC plates were adopted than when UT plates were adopted. Most mutants involved in overlaps harbored alterations at codons previously shown to confer a weak to modest elevation of echinocandin MICs (R647 and R1361 in *C. albicans*, D666 in *C. glabrata*, and P663 in *C. krusei*) (9–11), yet one *C. tropicalis* isolate harboring an alteration at the F650 codon was frequently missed as a non-wild-type isolate for rezafungin with all plates and also for anidulafungin with the Nunc, Greiner, and Costar UT plates. Overall, more overlaps between wild-type and non-wild-type isolates were observed for rezafungin than for anidulafungin. This is due to either technical issues associated with antifungal susceptibility testing (AFST) or, and potentially more likely, the fact that the *in vitro* activity of rezafungin is less affected by these alterations. Differential activity has previously been observed across the three licensed echinocandins. For example, some Fks alterations affect the activity of anidulafungin and caspofungin but not that of micafungin against *C. glabrata*, and others affect susceptibility much more in *C. krusei* than in *C. albicans* (12, 13). Therefore, it remains to be understood how many of these overlaps between MICs for mutants and wild-type isolates should be categorized as errors for rezafungin.

This study has limitations. First and foremost, not all plates were tested in both labs. Other factors may affect the MIC determination, including the potency of the powder, the means of storage of the plates, and, potentially, the loss of compound bound in plastics used for plate production (plates, pipette tips, etc.). However, our findings suggest that plate type rather than laboratory-specific patterns is responsible for the differential MICs observed. Second, the mutants included in the two laboratories were not identical, although most shared codons and alterations. Specifically, the MICs for one *C. tropicalis* isolate harboring an F650F/L alteration overlapped those for the *C. tropicalis* wild-type population in laboratory 1 for rezafungin and all plate types tested in laboratory 1 and for anidulafungin for the UT plates. This isolate was resequenced, and the Fks alteration was confirmed. Isolates with heterozygous alterations have previously been associated with MICs slightly lower than those for their counterparts with homozygous alterations (14). Laboratory 1 included three heterozygous mutants, while laboratory 2 included one. Consequently, we cannot exclude the possibility that the performance of the Falcon TC and UT plates as well as the Costar UT plates was overestimated compared to that of the other plates, as these were evaluated only in

TABLE 4 Fks mutant isolates included in the study from each of the two participating laboratories

Species	Amino acid alterations in Fks1/Fks2	
	Lab 1	Lab 2
C. albicans	Fks1, F641S, S645P, R647G, D648Y, and R1361H	Fks1, F641S, S645P, S645Y, D648Y, R1361H
C. glabrata	Fks1/2, Y1249-STOP/L664Q + Y658N[a]; Fks2, F659-DEL, F659S, S663P, S663F + L630Q,[a] and D666Y	Fks1, L630R; Fks2, F659-DEL, S663P, L664R, D666E
C. krusei	Fks1, F655F/C, L658W + L701M[a], D662Y, and R1368G	Fks1, F655I, L658W, P663Q, R1368G
C. tropicalis	Fks1, F650F/L, F650F/C, F650S, S654P, and S654S/P	Fks1, F650L, F650S, S654F, S654F, S654S/P

[a]Amino acid alteration at a codon outside the hot spot regions.

laboratory 2. Nevertheless, if the *C. tropicalis* isolate harboring an F650F/L alteration is excluded, the overall conclusions remain unchanged, in that fewer overlaps were found for TC plates than for UT plates and fewer overlaps were found for anidulafungin than for rezafungin. This observation supports a recommendation of favoring TC plates.

In conclusion, for both rezafungin and anidulafungin AFST, TC plates were preferable. Although not shown in this study, we observed air bubbles in UT plates (most notably, for Costar UT plates), which might complicate endpoint reading, particularly if the plates were also used for mold AFST with visual endpoint reading. Plate-to-plate variation was observed and correlated with the interlaboratory variation observed in our recent multicenter study (5), and again, the variation was the most pronounced for the most susceptible species (*C. albicans*), possibly because low concentrations are more susceptible to drug binding to plastics. We therefore believe that a careful choice of microtiter plates is important for AFST standardization.

MATERIALS AND METHODS

Isolates. The following six EUCAST control strains were tested repeatedly in both laboratories: *Candida albicans* CNM-CL F8555, ATCC 64548, and ATCC 64550; *Candida krusei* CNM-CL-3403 and ATCC 6258; and *Candida parapsilosis* ATCC 22019. Subsequently, in each laboratory, five to six wild-type isolates of each of the following species were included: *C. albicans*, *Candida glabrata*, *C. krusei*, *Candida tropicalis*, and *C. parapsilosis*. In addition, four (*C. krusei*) to five (*C. albicans*, *C. glabrata*, and *C. tropicalis*) *fks* mutant isolates were included in each laboratory, as detailed in Table 4.

Eight flat-bottom polystyrene plates were included: (i) Thermo Scientific (Nunc) 96F untreated plates (catalog no. 243656; Sigma-Aldrich Denmark A/S, Copenhagen, Denmark), (ii) Thermo Scientific (Nunc) 96F cell culture-treated plates (catalog no. 167008; Sigma-Aldrich), (iii) Greiner non-tissue culture-treated plates (catalog no. 655161; Sigma-Aldrich), (iv) Greiner tissue culture-treated plates (catalog no. 655180; Sigma-Aldrich), (v) Costar untreated plates (catalog no. 3370 from Sigma-Aldrich or catalog no. 07-200-656 from Fisher Scientific), (vi) Costar tissue culture-treated plates (catalog no. CLS3596; Sigma-Aldrich), (vii) Falcon untreated plates (catalog no 351172; Cultek), and (viii) Falcon tissue culture-treated plates (catalog no. 353072; Cultek). The first three plate brands were included in laboratory 1, the fourth and fifth plate brands were included in both laboratories, and the last three plate brands were included in laboratory 2 only.

Susceptibility testing. EUCAST susceptibility testing was performed according to the E.Def 7.3.1 reference method (15). Rezafungin (Cidara Therapeutics, San Diego, CA) and anidulafungin (catalog no. ADF00-100; Molcan, ON, Canada) were stored in aliquots of pure substance at −80°C. The compounds were dissolved in dimethyl sulfoxide (stock concentration, 5,000 mg/liter), and the MIC values were determined using 2-fold dilutions over the following concentration ranges: 8 to 0.008 mg/liter for *C. parapsilosis* and mutant isolates and 0.5 to 0.0005 mg/liter for wild-type isolates of *C. albicans*, *C. glabrata*, *C. krusei*, and *C. tropicalis*. Microtiter plates prepared with 2-fold dilutions of antifungal compound were frozen at −80°C prior to use, as is part of routine practice in clinical microbiology laboratories.

Data management and statistics. Mean MICs and ranges for each species and plate type were estimated and compared for the QC strains and the wild-type isolates. The numbers of overlaps between wild-type and *fks* mutant isolates were determined and compared. Descriptive and comparative analyses were done. The significance of the differences between MIC values was determined by analysis of variance (ANOVA; with the Bonferroni *post hoc* test) or nonparametric tests. For comparison of two sets of samples, we used the *t* test on the $\log_2$-converted MIC values. The correlation between the MIC results obtained by plate type was evaluated for plates shared between the two laboratories by using Pearson's correlation coefficient (CC) and the intraclass correlation coefficient (ICC). The ICC was expressed to a maximum value of 1 and with the 95% confidence interval. In order to approximate a normal distribution, the MICs were transformed to $\log_2$ values. The ICC is a reverse measurement of the variability of the MIC values. The ICC was calculated using the formula (group mean square − error mean square)/(group mean square + error

mean square) and thus has a maximum value of 1 if there is a perfect correlation and a minimum value of −1 if there is a complete absence of a correlation. The ICC evaluates the correlation between values, offering a statistical significance, since it takes into account the number of cases and the absolute value of the counting. The ICC is a scales analysis and exhibits the highest statistical power for correlation studies. Statistical analysis was performed with IBM SPSS Statistics (version 20.0) software (SPSS Iberica, Madrid, Spain). A P value of <0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00659-19.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

ACKNOWLEDGMENTS

We thank Birgit Brandt, Teresa Merino, and Cristina Armentia for their excellent technical assistance.

This study was supported by an unrestricted grant from Cidara Therapeutics.

The funder had no influence on the study design or on the analysis of the results.

Outside this work, we have the following potential conflicts to declare: M.C.A. has, over the past 5 years, received research grants (paid to the institution) or speaker honoraria (personal fee) from Amplyx, Astellas, Basilea, Cidara, F2G, Gilead, MSD, Novartis, Pfizer, and T2Biosystems. She is the current chairman of the EUCAST-AFST and has previously served on advisory boards for MSD (until 2014) and Pfizer (until 2012). K.M.J. has received meeting travel grants from Amplyx, MSD, and F2G. R.K.H. has received a research grant from Gilead and travel grants from Gilead, Astellas, MSD, and Pfizer. M.C.-E. has received grant support from Astellas Pharma, bioMérieux, Gilead Sciences, Merck Sharp & Dohme, Pfizer, Schering Plough, CIDARA, Amplyx, F2G, Scynexis, Soria Melguizo SA, Ferrer International, the European Union, the ALBAN program, the Spanish Agency for International Cooperation, the Spanish Ministry of Culture and Education, the Spanish Health Research Fund, the Instituto de Salud Carlos III (Spanish Ministry of Economy and Competitiveness), the Ramón Areces Foundation, and the Mutua Madrileña Foundation. He has been an advisor/consultant to the Panamerican Health Organization, Astellas Pharma, Gilead Sciences, Merck Sharp & Dohme, Pfizer, and Schering Plough. He has been paid for talks on behalf of Gilead Sciences, Merck Sharp & Dohme, Pfizer, Astellas Pharma, and Schering Plough. O.Z. has received grants and honoraria over the past 5 years from Gilead.

REFERENCES

1. Sandison T, Ong V, Lee J, Thye D. 2016. Safety and pharmacokinetics of CD101 IV, a novel echinocandin, in healthy adults. Antimicrob Agents Chemother 61:e01627-16. https://doi.org/10.1128/AAC.01627-16.
2. Krishnan BR, James KD, Polowy K, Bryant BJ, Vaidya A, Smith S, Laudeman CP. 2017. CD101, a novel echinocandin with exceptional stability properties and enhanced aqueous solubility. J Antibiot (Tokyo) 70:130–135. https://doi.org/10.1038/ja.2016.89.
3. Lakota EA, Bader JC, Ong V, Bartizal K, Miesel L, Andes DR, Bhavnani SM, Rubino CM, Ambrose PG, Lepak AJ. 2017. Pharmacological basis of CD101 efficacy: exposure shape matters. Antimicrob Agents Chemother 61:e00758-17. https://doi.org/10.1128/AAC.00758-17.
4. Pfaller MA, Messer SA, Rhomberg PR, Jones RN, Castanheira M. 2016. Activity of a long-acting echinocandin, CD101, determined using CLSI and EUCAST reference methods, against Candida and Aspergillus spp., including echinocandin- and azole-resistant isolates. J Antimicrob Chemother 71:2868–2873. https://doi.org/10.1093/jac/dkw214.
5. Arendrup MC, Meletiadis J, Zaragoza O, Jørgensen KM, Marcos-Zambrano LJ, Kanioura L, Cuenca-Estrella M, Mouton JW, Guinea J. 2018. Multicenter determination of rezafungin (CD101) susceptibility of Candida species by the EUCAST method. Clin Microbiol Infect 24:1200–1204. https://doi.org/10.1016/j.cmi.2018.02.021.
6. Fothergill AW, McCarthy DI, Albataineh MT, Sanders C, McElmeel M, Wiederhold NP. 2016. Effects of treated versus untreated polystyrene on caspofungin in vitro activity against Candida species. J Clin Microbiol 54:734–738. https://doi.org/10.1128/JCM.02659-15.
7. Howard SJ, Lass-Flörl C, Cuenca-Estrella M, Gomez-Lopez A, Arendrup MC. 2013. Determination of isavuconazole susceptibility of Aspergillus and Candida species by the EUCAST method. Antimicrob Agents Chemother 57:5426–5431. https://doi.org/10.1128/AAC.01111-13.
8. Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, Canton E, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Fothergill A, Fuller J, Gaustad P, Gonzalez GM, Guarro J, Lass-Flörl C, Lockhart SR, Meis JF, Moore CB, Ostrosky-Zeichner L, Pelaez T, Pukinskas S, St-Germain G, Szeszs MW, Turnidge J. 2013. Interlaboratory variability of caspofungin MICs for Candida spp. using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? Antimicrob Agents Chemother 57:5836–5842. https://doi.org/10.1128/AAC.01519-13.
9. Arendrup MC, Perlin DS. 2014. Echinocandin resistance: an emerging clinical problem? Curr Opin Infect Dis 27:484–492. https://doi.org/10.1097/QCO.0000000000000111.
10. Staab JF, Neofytos D, Rhee P, Jiménez-Ortigosa C, Zhang SX, Perlin DS, Marr KA. 2014. Target enzyme mutations confer differential echinocandin susceptibilities in Candida kefyr. Antimicrob Agents Chemother 58:5421–5427. https://doi.org/10.1128/AAC.00096-14.
11. Perlin DS. 2015. Mechanisms of echinocandin antifungal drug resistance. Ann N Y Acad Sci 1354:1–11. https://doi.org/10.1111/nyas.12831.
12. Arendrup MC, Perlin DS, Jensen RH, Howard SJ, Goodwin J, Hope W. 2012. Differential in vivo activities of anidulafungin, caspofungin, and micafungin against Candida glabrata isolates with and without FKS resistance mutations. Antimicrob Agents Chemother 56:2435–2442. https://doi.org/10.1128/AAC.06369-11.
13. Jensen RH, Justesen US, Rewes A, Perlin DS, Arendrup MC. 2014. Echi-

nocandin failure case due to a previously unreported FKS1 mutation in Candida krusei. Antimicrob Agents Chemother 58:3550–3552. https://doi.org/10.1128/AAC.02367-14.
14. Jensen RH, Johansen HK, Arendrup MC. 2013. Stepwise development of a homozygous S80P substitution in Fks1p, conferring echinocandin resistance in Candida tropicalis. Antimicrob Agents Chemother 57:614–617. https://doi.org/10.1128/AAC.01193-12.
15. Arendrup MC, Meletiadis J, Mouton JW, Guinea J, Cuenca-Estrella M, Lagrou K, Howard SJ, Arendrup MC, Meletiadis J, Howard SJ, Mouton J, Guinea J, Lagrou K, Arikan-Akdagli S, Barchiesi F, Hamal P, Järv H, Lass-Flörl C, Mares M, Matos T, Muehlethaler K, Rogers TR, Torp Andersen C, Verweij P. 2016. EUCAST technical note on isavuconazole breakpoints for Aspergillus, itraconazole breakpoints for Candida and updates for the antifungal susceptibility testing method documents. Clin Microbiol Infect 22:571.e1–e4. https://doi.org/10.1016/j.cmi.2016.01.017.