J Antimicrob Chemother
https://doi.org/10.1093/jac/dkac373

Journal of Antimicrobial Chemotherapy

Multicentre validation of a modified EUCAST MIC testing method and development of associated epidemiologic cut-off (ECOFF) values for rezafungin

Maiken Cavling Arendrup [1,2,3]*, Sevtap Arikan-Akdagli [4]†, Mariana Castanheira [5]†, Jesus Guinea [6,7,8]†, Jeffrey B. Locke [9]†, Joseph Meletiadis [10,11]† and Oscar Zaragoza [12,13]†

[1]Unit of Mycology, Statens Serum Institut, Copenhagen, Denmark; [2]Department of Clinical Microbiology, Rigshospitalet, Copenhagen, Denmark; [3]Department of Clinical Medicine, University of Copenhagen, Copenhagen, Denmark; [4]Unit of Mycology, Department of Medical Microbiology, Hacettepe University Medical School, Ankara, Turkey; [5]JMI Laboratories, North Liberty, IA, USA; [6]Clinical Microbiology and Infectious Diseases, Hospital General Universitario Gregorio Marañón, Madrid, Spain; [7]Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain; [8]CIBER Enfermedades Respiratorias-CIBERES (CB06/06/0058), Madrid, Spain; [9]Cidara Therapeutics Inc., San Diego, CA, USA; [10]Clinical Microbiology Laboratory, Attikon University Hospital, National and Kapodistrian University of Athens, Athens, Greece; [11]Department of Medical Microbiology and Infectious Diseases, Erasmus MC, Rotterdam, the Netherlands; [12]Mycology Reference Laboratory, National Centre for Microbiology, Health Institute Carlos III, Majadahonda, Madrid, Spain; [13]Center for Biomedical Research in Network in Infectious Diseases (CIBERINFEC-CB21/13/00105), Instituto de Salud Carlos III, Madrid, Spain

*Corresponding author. E-mail: maca@ssi.dk
†Contributed equally to the study.

Received 2 August 2022; accepted 11 October 2022

Objectives: Rezafungin EUCAST MIC testing has been associated with notable inter-laboratory variation, which prevented ECOFF setting for *C. albicans*. We assessed *in vitro* susceptibility and reproducibility for a modified EUCAST methodology and established associated wild-type upper limits (WT-ULs).

Methods: MICs against 150 clinical *Candida* isolates (six species), molecularly characterized fks mutants ($n=13$), and QC strains ($n=6$) were determined at six laboratories according to E.Def 7.3 but using Tween 20 supplemented medium. WT-ULs were determined using the derivatization method, the ECOFFinder programme and visual inspection. Consensus WT-ULs were determined.

Results: The laboratory- and species-specific MIC distributions were Gaussian with >99.5% MICs within four 2-fold dilutions except for *C. parapsilosis* (92.8%). The following consensus WT-UL were determined: *C. albicans* 0.008 mg/L; *C. dubliniensis* and *C. glabrata* 0.016 mg/L; *C. krusei* and *C. tropicalis* 0.03 mg/L; and *C. parapsilosis* 4 mg/L. Adopting these WT-UL, six clinical isolates were non-wild-type, five of which harboured Fks alterations. For 11/13 mutants, all 670 MICs were categorized as non-wild-type whereas MICs for *C. glabrata* Fks2 D666Y and *C. tropicalis* Fks1 R656R/G overlapped with the corresponding wild-type distributions. Repeat testing of six reference strains yielded 98.3%–100% of MICs within three 2-fold dilutions except for *C. albicans* CNM-CL-F8555 (96%) and *C. parapsilosis* ATCC 22019 (93.3%).

Conclusions: The modified EUCAST method significantly improved inter-laboratory variation, identified wild-type populations and allowed perfect separation of wild-type and *fks* mutants except for two isolates harbouring weak mutations. These consensus WT-UL have been accepted as ECOFFs and will be used for rezafungin breakpoint setting.

Introduction

Rezafungin (CD101) is a novel echinocandin with a half-life of approximately 130 hours, which allows a once-weekly dosing regimen for invasive infections.[1–4] The safety, tolerability and efficacy of rezafungin compared to caspofungin followed by fluconazole have been evaluated in a phase-II study (NCT02734862, STRIVE)[5] and a randomized double-blind phase-III trial (NCT03667690, ReSTORE) (Study of Rezafungin Compared to Caspofungin in Subjects With Candidemia and/or Invasive

Candidiasis—Full Text View—ClinicalTrials.gov accessed 18 March 2022). The drug target and binding site are the same as for anidulafungin, caspofungin and micafungin, and mutations in one of the hot spot regions of the *fks* target gene(s) affect susceptibility to all four echinocandins in most cases although subtle differences may occur.[6–9]

CLSI has set provisional epidemiological cut-off values (ECVs in CLSI terminology, ECOFFs in EUCAST terminology) for rezafungin and provisional clinical breakpoints based on multicentre data generated using plates prepared in-house. In addition, CLSI target MIC ranges for quality control strains have been set using commercially produced Trek panels (Table S1, available as Supplementary data at *JAC* online).[10] A recent study adopted the breakpoints on a global collection of 1427 non-duplicate invasive *Candida* isolates and found all *Candida albicans*, *Candida dubliniensis*, *Candida krusei* and *Candida tropicalis* isolates susceptible, as were 98.3% of the *Candida glabrata* and 99.6% of *Candida parapsilosis* isolates.[9]

EUCAST also sets ECOFFs and clinical breakpoints for antifungals based on multicentre MIC data generated in laboratories with in-house prepared microtitre susceptibility testing plates. This approach facilitates recognition of any notable inter-laboratory variation related to technical issues including brand and type of trays, reservoirs and medium, which needs attention. A four-centre study in 2018 reported an unacceptable inter-laboratory variation for rezafungin EUCAST MICs against *C. albicans*.[11] In detail, one of the criteria EUCAST has set for qualifying MIC distributions for aggregation is that the modal MIC of each distribution shall fall within ±1 dilution of the most common modal MIC across the individual distributions and this criterion was not met for the four *C. albicans* distributions.[12] A subsequent study confirmed the findings and demonstrated that choice of microtitre plate notably affected the MIC determinations particularly for *C. albicans*, the most susceptible of the studied species.[13] Of note, this was also observed for the comparator echinocandin anidulafungin. It has been shown that some hydrophobic compounds stick to plastic in antifungal susceptibility RPMI-1640 test medium where the DMSO concentration is 'only' 1% (final concentration 0.5% after inoculation).[14] This can lead to concentrations in the wells that are lower than the target drug concentration due to drug loss in plastics used during microtitre plate preparation such as interim tubes, pipette tips and multichannel reservoirs or to the plastic in the plate itself. Conversely, it can lead to concentrations in the wells that are higher than the target concentration if serial dilution is used due to release of bound agent from the pipette tip in subsequent steps if tip changes are not performed. This challenge is not unique to antifungals. Some antibacterials stick heavily and variably to plastic.[15–17] For example, less than 10% of [^{14}C]oritavancin was recovered in broth in microtitre plates at 1 h when [^{14}C]oritavancin was tested at 1 mg/L. Furthermore, proportionately greater losses were observed at lower oritavancin concentrations, suggesting saturable binding of oritavancin to surfaces. This was prevented by supplementation of the growth medium with 0.002% Tween 80 (also known as polysorbate 80 and T80).[16] Although modifications of reference methods are in general not preferred, it may be essential for some agents to achieve a sufficiently high inter-laboratory reproducibility and good separation of wild-type from mutant isolates.

On this background, a modified EUCAST E.Def 7.3 method, in which the medium was supplemented with Tween 20 (also known as polysorbate 20 and T20) at a final concentration of 0.002% (in inoculated wells) was developed to prevent drug binding and drug loss in interim plastic ware and microtitre plates.[18] Tween 20 was preferred over Tween 80 because Tween 20 is already in use in mycology laboratories for inoculum preparations for mould susceptibility testing.[19] The purpose of this study was to evaluate this method in a multicentre study with the intention of generating a robust and reproducible method that allows to generate reliable MIC data that qualify for EUCAST ECOFFs setting.

Materials and methods

Study design and isolates

Six laboratories in Denmark, Greece, Spain, Turkey and the USA participated. Each laboratory tested three sets of isolates: first, 150 clinical isolates: at least 25 isolates per species of *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis* from local strain collections. Second, a shared blinded strain collection of 13 *fks* hotspot mutant isolates: *C. albicans* Fks1 D648Y and Fks1 S645P; *C. dubliniensis* Fks1 F641S and Fks1 R1361S; *C. glabrata* Fks2 D666Y, Fks2 F659-del, Fks2 F659S and Fks2 S663P; *C. krusei* Fks1 D662D/Y and Fks1 S659F; and *C. tropicalis* Fks1 F650S, Fks1 R656R/G and Fks1 S654S/P. Third, the following six quality control strains: *C. albicans* ATCC 64548, ATCC 64550 and CNM-CL-F8555; *C. krusei* ATCC 6258 and CNM-CL-3403; and *C. parapsilosis* ATCC 22019. The mutant and QC strains were tested at least 10 times in each of the participating laboratories, whereas the 150 local clinical strains were tested once. Species identification was done according to local standard procedures, including colony morphology and colour (CHROMagar Co., Paris, France), microscopic morphology, growth at 37°C and either 43 or 45°C, assimilation profile (API ID32C; bioMérieux), MALDI-TOF MS or molecular techniques (ITS-sequencing).[20]

Susceptibility testing and target gene sequencing

Rezafungin (Cidara Therapeutics, San Diego, CA, USA) pure substance was stored in aliquots at −70 to −80°C and stock solutions prepared in DMSO (5000 mg/L). MICs were determined following the E.Def 7.3 methodology with the modification that the double concentrated medium used for microtitre plate preparation was supplemented with Tween 20 at a concentration of 0.004%, resulting in a final concentration of 0.002% on inoculation of the microtitre plates with Candida in water. The medium was sterile filtered after Tween 20 addition. This step not only ensured the sterility but also proper mixing of Tween 20 into the medium. The susceptibility test microtitre plates were prepared using the ISO dilution method for hydrophobic agents, DMSO as solvent and a final 1:100 dilution of the 2-fold dilution series into double concentrated EUCAST medium supplemented with Tween 20.14 The final rezafungin concentrations studied ranged from 8 to 0.0001 mg/L. The concentrations were finally rounded up to be designated in two to four digits maximum. Ready-to-use microtitre plates were frozen at −70 to −80°C before use. The following brands of 96-well microtitre plates were used: Centre 1: Nunc MicroWell, Nunclon Delta-treated MicroWell plates, catalogue no. 167008; Thermo Fisher Scientific, Denmark, Centre 2: Thermo Scientific™ BioLite™ MicroWell plates, catalogue no. 130188, Thermo Fisher Scientific, Greece, Centre 3: Greiner bio-one, CELLSTAR, catalogue no. 655180; Frickenhausen, Germany, Centre 4: Brand CellGrade Microplate catalogue no. 781962, The US, Centre 5: Corning Costar, catalogue no. 3595, Merck KGaA, Darmstadt, Germany, Centre 6: Falcon® 96-well Clear Flat Bottom TC-treated Culture Microplate, Corning catalogue no. 353072. For clinical isolates with non-wild-type rezafungin

Table 1. Rezafungin MICs determined at six centres by the Tween 20-supplemented EUCAST E.Def 7.3 method against clinical isolates of the six most common *Candida* species

	MIC (mg/L)[a]																			
Species	0.0002	0.0005	0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	>8	Total	GM-MIC[b]	Modal MIC
C. albicans																				
Centre 1			11	12	2													25	0.0016	0.002
Centre 2		1	10	12	2				***1***									26	0.0015	0.002
Centre 3		1	21	3														25	0.0011	0.001
Centre 4	1	5	15	4	1													26	0.0010	0.001
Centre 5		2	13	7	3													25	0.0014	0.001
Centre 6			1	18	5	3												27	0.0026	0.002
Total	1	9	71	56	13	3			***1***									154	0.0014	0.001
C. dubliniensis																				
Centre 1				5	19	1												25	0.0036	0.004
Centre 2				1	8	13	2											24	0.0063	0.008
Centre 3				10	13	2												25	0.0032	0.004
Centre 4	1	2	4	7	12													26	0.0021	0.004
Centre 5			1	4	19	1												25	0.0035	0.004
Centre 6			3	9	10	4												26	0.0030	0.004
Total	1	2	8	36	81	21	2											151	0.0034	0.004
C. glabrata																				
Centre 1					12	13												25	0.0057	0.008
Centre 2						22	1	1				***1***						25	0.0087	0.008
Centre 3					10	15												25	0.0061	0.008
Centre 4				2	8	16												26	0.0058	0.008
Centre 5					11	12	2											25	0.0062	0.008
Centre 6						22	5											27	0.0091	0.008
Total				2	41	100	8	1				***1***						153	0.0068	0.008
C. krusei																				
Centre 1						4	19	1	***1***									25	0.0155	0.016
Centre 2				1		3	20		***1***									25	0.0135	0.016
Centre 3						25												25	0.0080	0.008
Centre 4						15	13											28	0.0107	0.008
Centre 5						11	10	4										25	0.0130	0.008
Centre 6						1	24	1										26	0.0160	0.016
Total				1		59	86	6	***2***									154	0.0124	0.016
C. parapsilosis																				
Centre 1													6	11	8			25	2.1140	2
Centre 2													16	6	2		***1***	25	1.3348	1
Centre 3											7	11	7					25	0.5000	0.5
Greiner repeat										1	4	8	11	1				25	0.6071	1
Nunc repeat											3	9	10	3				25	0.7170	1
Centre 4											5	14	7					26	0.5274	0.5
Centre 5										1	5	15	2	2				25	0.4863	0.5
Centre 6												3	17	7				27	1.1081	1

Continued

Table 1. *Continued*

Species	MIC (mg/L)[a]																GM-MIC[b]	Modal MIC
	0.0002	0.0005	0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	>8	Total		
Total (excl. repeat test)										1	17	43	55	26	10		**1**	153	0.8587	0.5
Total (incl. repeat test)										2	24	60	76	30	10		**1**	203	0.8040	1
C. tropicalis																				
Centre 1					7	14	4											25	0.0074	0.008
Centre 2					1	18	6											25	0.0092	0.008
Centre 3				1	18	6												25	0.0046	0.004
Centre 4					9	16	1											26	0.0064	0.008
Centre 5				3	18	4												25	0.0041	0.004
Centre 6					1	4	21	1										27	0.0140	0.016
Total				4	54	62	32	1										153	0.0070	0.008

Isolate numbers that are underlined indicate isolates that are classified as non-wild-type isolates adopting the consensus WT-UL in Table 2). Numbers in ***bold italic*** font represent isolates that harbour one or more Fks alterations. Normal font indicates MIC values within the wild-type population (Table 2).

[a]The concentrations were finally rounded up to be designated in two to four digits maximum.

[b]GM-MIC: Geometric MIC.

MICs according to the consensus wild-type upper MIC limit (WT-UL) established in this study (see next), susceptibility to anidulafungin and micafungin was determined using the reference method EUCAST E.Def 7.3 for comparison and echinocandin target gene sequencing was performed (*fks1* and for *C. glabrata* also *fks2*).[19]

Data management

Geometric mean MICs (GM-MICs) and modal MICs (most common MIC) were determined for each distribution and aggregated distributions. The variation across centres was evaluated for each species as the number of dilution steps between the GM-MIC for each centre and GM-MIC for the aggregated distribution as follows: the mean of the numeric values of ($\log_2$ GM-MIC$_{\text{single-centre distribution}}$ − $\log_2$ GM-MIC$_{\text{aggregated distribution}}$). MIC distributions were regarded qualified for aggregation and subsequent WT-UL determination (defined as the MIC value where the wild-type distribution ends), when the distribution was unimodal and the modal MIC was within ±1 dilution of the most common MIC for that particular species as described in the EUCAST.[12] WT-ULs were determined using the derivatization method, the ECOFFinder program with 97.5%–99.9% of the modelled distributed (available at EUCAST: ECOFFinder program updated accessed 10-06-2022), and visually for aggregated data set.[21,22] Consensus WT-UL values were defined by the authors and have subsequently been accepted as formal EUCAST epidemiologic cut-off values (ECOFFs) by the EUCAST Steering Committee.

Results

Susceptibility data

Rezafungin displayed a species-specific *in vitro* activity with the lowest MICs observed against clinical *C. albicans* and the highest against *C. parapsilosis* (Table 1). The individual laboratory- and species-specific MIC distributions were unimodal and Gaussian with >99.5% of MICs within four dilutions for all species except *C. parapsilosis* where 92% of MICs fell within the four-dilution range. The centre- and species-specific modal MICs fell within ±1 dilution of the most common modal MIC, except for *C. parapsilosis* where the most common modal MIC was 0.5 mg/L but 2 mg/L for centre 1 (Table 1). The mean difference between the species- and centre specific GM-MIC and the GM-MIC of the aggregated distributions was low (0.26–0.49 2-fold dilutions) although larger for *C. parapsilosis* (0.77 2-fold dilutions) (Table 2). To further study, the variation for *C. parapsilosis*, centre 3 (representing a low MIC centre together with centres 4 and 5) repeated *C. parapsilosis* testing on two different brands of plates. On both occasions, a modal MIC of 1 mg/L was found compared to 0.5 mg/L at initial testing (Table 1). Including these data sets, the most common modal MIC for *C. parapsilosis* was 1 mg/L, the mean difference between the species- and centre- specific GM-MIC and the GM-MIC of the aggregated distributions was lower (0.65 2-fold dilutions) and all modal MIC values within ±1 dilution of the most common modal MIC (Tables 1 and 2).

Wild-type populations

Next, WT-UL values were determined using the derivatization method, the ECOFFinder programme including 97.5%, 99%, 99.5% and 99.9%, respectively, of the modelled aggregated populations and a visual inspection. The determined values were either identical across all methods (*C. glabrata* 0.016 mg/L and *C. krusei* 0.03 mg/L) or fell within two or three (*C. parapsilosis*

Table 2. Rezafungin susceptibility, WT-UL values determined using the derivatization method, the ECOFFinder programme and visually and the consensus WT-UL, subsequently accepted as EUCAST ECCOFs

Species (*n* distributions)	GM-MIC (mg/L)	Mean number of 2-fold dilution steps between centre specific GM-MIC and the mean GM-MIC (mg/L)	WT-UL (mg/L) determined by the derivatization method	WT-UL (mg/L) determined including 97.5%–99.9% of the modelled population				WT-UL visual (mg/L)	ECOFF (mg/L)
				WT-UL 97.5%	WT-UL 99%	WT-UL 99.5%	WT-UL 99.9%		
C. albicans (6)	0.0014	0.35	0.004	0.004	0.004	0.004	0.008	0.008	0.008
C. dubliniensis (6)	0.0034	0.34	0.008	0.008	0.008	0.016	0.016	0.016	0.016
C. glabrata (6)	0.0068	0.26	0.016	0.016	0.016	0.016	0.016	0.016	0.016
C. krusei (6)	0.1240	0.28	0.03	0.03	0.03	0.03	0.03	0.03	0.03
C. parapsilosis (6)	0.8587	0.77	2	4	4	4	8	4	4
C. parapsilosis (8)[a]	0.6483	0.65	2	4	4	4	8	4	4
C. tropicalis (6)	0.0070	0.49	0.03	0.016	0.03	0.03	0.03	0.03	0.03

[a]including the repeat testing at centre 3.

Table 3. Extended analyses of six local clinical isolates for which the initial rezafungin MIC was elevated compared to the main population

Isolates with elevated rezafungin MICs	Initial rezafungin MIC (mg/L)	Repeated rezafungin MIC (mg/L)		Anidulafungin MIC (mg/L) Centre 1	Micafungin MIC (mg/L) Centre 1	Mutant Fks region	AA substitution
		Centre 2	Centre 1				
C. albicans AUH1202	**0.06**	**0.06**	**0.125**	0.03	**0.06**	Fks1 HS2	D1337Y
C. glabrata AUH379	**0.5**	**0.5**	**0.06**	**1**	**0.25**	Fks2 HS1	S663F
C. glabrata AUH1740	**0.03**	**0.03**	0.016	0.03	0.016	None	None
C. krusei AUH1940	**0.06**	**0.06**	**0.06**	**0.125**	**2**	Fks1 HS1	S659P
C. krusei SSI-77.20	**0.06**	ND	ND	0.06	**0.5**	Fks1 HS1	S659S/P
C. parapsilosis AUH1957	**>8**	**>8**	**>8**	**>4**	**>4**	Fks1 HS1	F652S

Non-wild-type MICs for rezafungin, anidulafungin and micafungin are indicated in **bold** font.
ND: not done.

specifically) dilutions (Table 2). Closest agreement between visual and ECOFFinder WT-UL was found using the ECOFFinder 99.9% value except for *C. parapsilosis* where WT-UL values were 4 mg/L and identical across ECOFFinder values using 97.7% to 99.5% and the visual WT-UL. For wild-type versus non-wild-type classification the following ECOFFs were approved: *C. albicans* 0.008 mg/L; *C. dubliniensis* and *C. glabrata* 0.016 mg/L; *C. krusei* and *C. tropicalis* 0.03 mg/L; and *C. parapsilosis* 4 mg/L. Adopting these values for rezafungin MIC interpretation, six isolates were classified as non-wild-type isolates. Target gene sequencing revealed hot spot alterations and elevated MICs for anidulafungin or micafungin in five of these isolates. These included one *C. albicans* [rezafungin MIC 0.06 mg/L (three dilutions above the ECOFF), Fks1: D1337Y], one *C. glabrata* [rezafungin MIC 0.5 mg/L (five dilutions above the ECOFF), Fks2: S663F], two *C. krusei* [both rezafungin MIC 0.06 mg/L (both one dilution above the ECOFF), Fks1 S659S/P and Fks1 S659P, respectively] and one *C. parapsilosis* isolate [rezafungin MIC >8 mg/L (≥1 dilution above the ECOFF), Fks1 F652S] (Table 3). The remaining *C. glabrata* isolate determined with rezafungin MIC of 0.03 mg/L (one dilution above the ECOFF) in centre 2 was found to be rezafungin, anidulafungin and micafungin and target gene wild-type in centre 1 (Table 3).

Fks *mutant strains*

Susceptibility testing was performed repeatedly (≥10 times/laboratory) for 13 *fks* mutant strains including two *C. albicans*, two *C. dubliniensis*, four *C. glabrata*, two *C. krusei* and three *C. tropicalis* selected to represent both weak and strong mutants (Table 4). For 11 out of the 13 mutants, all 670 MIC determinations were above the species-specific ECOFF values. In contrast, for two mutants (*C. glabrata* Fks2 D666Y and *C. tropicalis* Fks1 R656R/G) the MIC range overlapped the ECOFF resulting in 17/

Table 4. Rezafungin susceptibility testing (792 MICs in total) of 13 molecularly characterized *fks* mutants

	MIC (mg/L)																
Species AA alteration	0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	>8	Total	GM-MIC
C. albicans Fks1 D648Y																	
Centre 1							7	3								10	0.0748
Centre 2							1	9								10	0.1166
Centre 3					5	5										10	0.0219
Centre 4					2	7	1									10	0.0280
Centre 5						3	5	3								11	0.0607
Centre 6						3	7									10	0.0487
Total for the mutant					7	18	21	15								61	
Total for wild-type isolates	81	56	13	3												154	
C. albicans Fks1 S645P																	
Centre 1								4	3	3						10	0.2333
Centre 2								8	2							10	0.1436
Centre 3							4	6								10	0.0932
Centre 4							3	7								10	0.0975
Centre 5								1	7	2	1					11	0.3020
Centre 6								1	9							10	0.2323
Total for the mutant							7	27	21	5	1					61	
Total for wild-type isolates	81	56	13	3												154	
C. dubliniensis Fks1 F641S																	
Centre 1							2	8								10	0.1079
Centre 2							1	9		1						11	0.1166
Centre 3							10		1							11	0.0600
Centre 4							8	2								10	0.0689
Centre 5							9	2								11	0.0686
Centre 6							9	1								10	0.0643
Total for the mutant							39	22	1	1						63	
Total for wild-type isolates	11	36	81	21	2											151	
C. dubliniensis Fks1 R1361S																	
Centre 1									5	5						10	0.3536
Centre 2										9						9	0.5000
Centre 3								1	8							9	0.2333
Centre 4									10							10	0.2500
Centre 5								1	6	4						11	0.3020
Centre 6									7	3						10	0.3078
Total for the mutant								2	36	21						59	
Total for wild-type isolates	11	36	81	21	2											151	
C. glabrata Fks2 D666Y																	
Centre 1					5	5										10	0.0219
Centre 2						10										10	0.0313
Centre 3					5	5										10	0.0219
Centre 4					4	6										10	0.0227
Centre 5					3	7	1									11	0.0269
Centre 6						8	2									10	0.0345
Total for the mutant					17	41	3									61	
Total for wild-type isolates		2	41	100	8	1										153	
C. glabrata Fks2 F659-del																	
Centre 1						6	3	1								10	0.0426
Centre 2							6	4								10	0.0825
Centre 3						4	6									10	0.0455
Centre 4						1	9									10	0.0560
Centre 5							7	4								11	0.0784

Continued

Table 4. *Continued*

	MIC (mg/L)																
Species AA alteration	0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	>8	Total	GM-MIC
Centre 6											2	8				10	1.7411
Total for the mutant						11	31	9			2	8				61	
Total for wild-type isolates		2	41	100	8											153	
C. glabrata Fks2 F659S																	
Centre 1						10										10	0.0300
Centre 2						2	8									10	0.0544
Centre 3						10										10	0.0300
Centre 4						10										10	0.0300
Centre 5						5	6									11	0.0438
Centre 6											6	4				10	1.3195
Total for the mutant						37	14				6	4				61	
Total for wild-type isolates		2	41	100	8											153	
C. glabrata Fks2 S663P																	
Centre 1								5	3	2						10	0.2031
Centre 2											1	7	2			10	2.1435
Centre 3											10					10	1.0000
Centre 4									1	2	7					10	0.7579
Centre 5												8	3			11	2.4162
Centre 6											4	6				10	1.5157
Total for the mutant								5	4	4	22	21	5			61	
Total for wild-type isolates		2	41	100	8											153	
C. krusei Fks1 D662D/Y									1	2	22	21	5				
Centre 1														8	2	10	9.1896
Centre 2															10	10	16.0000
Centre 3											1	6	3			10	2.2974
Centre 4									6					1	3	10	0.4102
Centre 5								3	5	2						10	0.2333
Centre 6														10		10	8.0000
Total for the mutant								3	11	2	1	6	3	19	15	60	
Total for wild-type isolates		1	0	59	86	6										154	
C. krusei Fks1 S659F																	
Centre 1									9	1						10	0.2679
Centre 2									10							10	0.2500
Centre 3							1	9								10	0.1162
Centre 4								9	1							10	0.1291
Centre 5								2	8	1						11	0.2347
Centre 6									2		1	3	1	3		10	2.0000
Total for the mutant							1	20	30	2	1	3	1	3		61	
Total for wild-type isolates		1	0	59	86	6										154	
C. tropicalis Fks1 F650S																	
Centre 1										10						10	0.5000
Centre 2										3	7					10	0.8123
Centre 3									8	2						10	0.2872
Centre 4									2	8						10	0.4353
Centre 5									3	8						11	0.4139
Centre 6										10						10	0.5000
Total for the mutant									13	41	7					61	
Total for wild-type isolates		4	54	62	32	1										153	
C. tropicalis Fks1 R656R/G																	
Centre 1						7	3									10	0.0369
Centre 2						9	1									10	0.0335

Continued

Table 4. *Continued*

	MIC (mg/L)																
Species AA alteration	0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	>8	Total	GM-MIC
Centre 3				8	2											10	0.0092
Centre 4					9	1										10	0.0161
Centre 5				1	3	5	2									11	0.0254
Centre 6						10										10	0.0300
Total for the mutant				9	14	32	6									61	
Total for wild-type isolates		4	54	62	32	1										153	
C. tropicalis Fks1 S654S/P																	
Centre 1								6		4						10	0.2176
Centre 2										10						10	0.5000
Centre 3								2	8							10	0.2176
Centre 4								4	6							10	0.1864
Centre 5									9	2						11	0.2836
Centre 6									5	5						10	0.3536
Total for the mutant								12	28	21						61	
Total for wild-type isolates		4	54	62	32	1										153	

The ECOFF (Table 2) defining the wild-type MICs as ≤X) are indicated by dashed vertical lines. The aggregated MIC distributions for the clinical wild-type isolates are included in a separate row for comparison in grey font (please note that these are truncated at 0.01 mg/L for *C. albicans* and *C. dubliniensis* but presented in full range in Table 1).

61 (27.9%) and 55/61 (90.2%) MIC determinations falling in the wild-type range, respectively. Overall, 720/792 (90.9%) MICs for *fks* mutants were in the non-wild-type MIC range.

QC strains

Six QC strains were tested repeatedly (10–18 times) in each of the six centres resulting in 69 to 73 MICs per strain (424 MICs in total, Table 5 and Table S2). All MICs against *C. krusei* ATCC 6258 and *C. krusei* CNM-CL-3403 and all but one MIC against *C. albicans* ATCC 64548 and *C. albicans* ATCC 64550 fell within three 2-fold dilutions. The MIC range expanded to include four dilutions against *C. albicans* CNM-CL-F8555 (ignoring one MIC determination) and against *C. parapsilosis* ATCC22019. The dominating MICs and MIC ranges are summarized in Table 5.

Discussion

A robust and reproducible reference testing method with associated MIC targets and ranges for relevant quality control strains is fundamental for clinical breakpoint setting. It is also fundamental for the development of reliable commercial tests that provide correct susceptibility classification of clinical isolates. EUCAST has set criteria for qualification of MIC distributions for aggregation and ECOFF setting.[12] In brief, at least a 100 isolates per species from at least five independent MIC distributions each including at least 15 isolates are required, and moreover the modal MIC of each distribution must be within ±1 dilution from the most common modal MIC. Whereas the four MIC distributions generated in a multicentre study using the EUCAST E.Def 7.3 method failed to meet these criteria for *C. albicans*, we here show that the dataset from six centres were in excellent agreement for the common *Candida* species and all distributions qualified for aggregation when testing was performed with Tween 20 supplemented medium.[11,13] We also showed that ECOFFs could be set following the EUCAST principles for ECOFF setting, and that five of six isolates classified as non-wild-type isolates indeed harboured target gene mutations. Finally, we challenged the method by repetitive testing of molecularly characterized *fks* mutant isolates that harboured strong (affecting phenylalanine and serine in hot spot 1) or weak mutations (aspartic acid hot spot 1 and arginine in hot spot 1 and 2). All strong and three of five weak mutants were consistently identified as non-wild-type isolates.

Rezafungin MIC testing using Tween 20 supplemented EUCAST growth medium confirmed previous findings using EUCAST E.Def 7.3 or CLSI M27 that *C. albicans* is the species most susceptible to rezafungin followed by *C. dubliniensis*, *C. glabrata*, *C. tropicalis* and *C. krusei* for which the MICs are two, three, three and four 2-fold dilutions higher than those for *C. albicans*, respectively.[9,11] *C. parapsilosis* was the least susceptible organism with MICs around 10 2-fold dilutions higher than for *C. albicans*. For agents that are highly potent at a mg/L basis, the MICs are very low and any drug loss due to binding to plastic or precipitation/aggregation in the medium may lower the 'free' available drug concentration and affect (increase) the MIC.[16] This will expectedly affect the most susceptible organisms at the highest extent as observed previously for rezafungin, anidulafungin, micafungin, fluconazole, isavuconazole and caspofungin.[11,14,23] This is in agreement with the observations that the modal MIC was three dilutions lower for *C. albicans*, two dilutions lower for *C. glabrata*, *C. krusei* and *C. tropicalis* and one dilution lower for *C. parapsilosis* compared to those obtained without Tween 20 in our previous multicentre study[11] and that the difference in modal MIC between *C. albicans* and *C. parapsilosis* was 10 2-fold dilutions using the Tween 20 supplemented medium,

Table 5. Aggregated rezafungin MICs against six quality control strains across the six centres

	Rezafungin MIC (mg/L)												Suggested targets		
Strain	0.0002	0.0005	0.001	0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	MIC (mg/L)	MIC range (mg/L)	Percentage of isolates
C. albicans																	
ATCC64548	0.6	**12.3**	**32.6**	**14.5**											0.001	0.0005–0.002	99
ATCC64550			1.0	**19.5**	**20.1**	**19.4**									0.004	0.002–0.008	98.3
CNM-CL-F8555		0.6	1.8	**17.5**	**29.3**	**10.8**									0.004	0.002–0.008	96
C. krusei																	
ATCC6258					**2.1**	**33.3**	**24.6**								0.008–0.016	0.004–0.03	100
CNM-CL-3403					0.6	**19.8**	**39.6**								0.008–0.016	0.004–0.03	99
C. parapsilosis																	
ATCC22019										**8.0**	**30.1**	**17.9**	**4.0**		0.25–0.5	0.125–1	100

Weighted values for number of isolates are used to balance centres with different number of replica values ($n = 10–18$ repetitions/centre per strain). Suggested MIC target (indicated by underlined font) and ranges (indicated by bold font) for QC strains based on the MIC data in this study (underlying MIC data is presented in Table S2).

versus eight and five 2-fold dilutions using the EUCAST and CLSI reference methods without Tween 20 supplementation, respectively.[9,11]

Whether the enhancement of MIC difference between high and low MIC species will improve detection of clinically resistant mutants remains to be investigated. However, we did not observe overlaps between MICs for mutants and wild-type organisms for mutants with amino acid substitutions affecting phenylalanine and serine in hot spot 1 using Tween 20-supplemented medium in contrast to our previous two-centre study when Tween 20 was not included.[13] Nevertheless, the majority (55/61) of the MICs obtained for the *C. tropicalis* strain harbouring an Fks1 R656R/G alteration and almost a third (17/61) of the MICs obtained against the *C. glabrata* strain harbouring an Fks2 D666Y alteration fell below the ECOFF. CLSI has recently released provisional clinical breakpoints for rezafungin, which are two dilutions above the ECVs for *C. albicans* and *C. glabrata*, and one dilution above the ECVs for *C. krusei* and *C. tropicalis*.[10] Adopting a similar approach to our data would classify 25/61 MICs against *C. albicans* harbouring Fks1 D648Y, all 61 MICs against *C. glabrata* Fks2 D666Y, 42/61 MICs against *C. glabrata* Fks2 F659-del, 51/61 *C. glabrata* Fks2 F659S, 1/61 *C. krusei* Fks1 S659F and all 61 MICs against *C. tropicalis* Fks1 R656R/G as susceptible. If setting clinical breakpoints that were only one dilution above the ECOFF for *C. albicans* and *C. glabrata*, these numbers would be 7/61 MICs against *C. albicans* harbouring Fks1 D648Y, 58/61 MICs against *C. glabrata* Fks2 D666Y, 11/61 MICs against *C. glabrata* Fks2 F659-del and 37/61 *C. glabrata* Fks2 F659S that would be classified as susceptible. If standard dosing is not sufficient to cover infections with these mutants this will confer a risk of therapeutic failures. However, a proposed breakpoint lower than the ECOFF will bisect the non-WT distributions and lead to random classification of wild-type isolates. Clinical data for the outcome of patients infected with such isolates on standard dosing is limited. One expanded access case report described suppression with rezafungin of an infection involving a multidrug resistant *C. glabrata* with a D666Y alteration in Fks2 and CLSI MIC above the CLSI ECV.[24] Moreover, the PK/PD AUC/CLSI MIC targets for stasis was remarkably lower for rezafungin against *C. glabrata* than against *C. albicans* in a mouse model including three wild-type *C. albicans* and one wild-type and two mutant *C. glabrata*.[25] Translated to clinical dosing this would suggest that the stasis target would be expected to be achieved with the standard dose of rezafungin against *C. albicans* isolates with CLSI MICs of ≤1 mg/L and against *C. glabrata* isolates with MICs of ≤16 mg/L.[25] Of note, resistance mutations in fungi may confer different levels of fitness cost.[8,26] Mutants were not included for *C. albicans* and virulence was not reported for the included *C. glabrata* mutants in the aforementioned target attainment study. Although data are promising, it remains to be fully understood how this may affect evaluations in animal models and whether it may cause differences in outcome across mutant isolates with the same elevated MIC in clinical practice.

Interpretation of susceptibility test results obtained with other methods including commercial tests requires that the results from such tests mirror those from the references method. As discussed before, method variation may not affect high and low MIC species to the same extent. Consequently, inter-laboratory and intra-laboratory variation may not be acknowledged unless

both low- and high-MIC QC strains are included in the routine validation of MIC testing. The MIC distributions for the six control strains were narrow and together with modal MICs spanning from 0.001 to 0.25 mg/L covered the MIC range for clinically relevant species and thus facilitate such validation.

In summary, rezafungin ECOFFs could be established with 0.008 mg/L for *C. albicans*, 0.016 mg/L for *C. dubliniensis* and *C. glabrata*, 0.03 mg/L for *C. krusei* and *C. tropicalis* and 4 mg/L for *C. parapsilosis*. Until official breakpoints, QC values and ranges are established, the ECOFFs will allow a reliable classification of the six most common *Candida* species as wild-type or non-wild-type, provided testing is validated for high and low ends of the concentration range. This can be done by ensuring that modal MIC of *C. albicans* ATCC 64548 and the widely used quality control strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 are on the defined target MIC and the MICs within the MIC ranges displayed in Table 5.

Acknowledgements

The authors wish to thank research technicians Birgit Brandt and Désiré Mageme Nahimana at the SSI, Ana Gómez at Gregorio Marañón Hospital, Maria Siopi at Attikon Hospital, Dilek Cakmak at Hacettepe University and Teresa Merino and Cristina de Armentia at the Health institute Carlos III for excellent technical assistance. In addition, we wish to thank Cecilia Carvalhaes, MD, PhD for coordinating the study at JMI Laboratories, Karin Meinike Jørgensen, PhD for coordinating the MIC testing at the SSI and Ilke Toker Onder, MD for coordinating the MIC testing at Hacettepe University.

Funding

This study was supported by an unrestricted grant from Cidara Therapeutics. The funder had no influence on the analysis of the results.

Transparency declaration

Outside this work, the authors have the following potential conflicts to declare: Ma.C.A. has received research grants/contract work (paid to the SSI) from Amplyx, Basilea, Cidara, F2G, Gilead, Novabiotics and Scynexis, and speaker honoraria (personal fee) from Astellas, Chiesi, Gilead, MSD and SEGES over the past 5 years. She is the current chairman of the EUCAST-AFST. S.A.A. received speaker honoraria from Gilead and travel grants from Astellas, Gilead and Pfizer over the past 5 years. J.G. is a permanent researcher contracted by Fundación para Investigación Sanitaria del Hospital Gregorio Marañón. J.G. has received funds for participating at educational activities organized on behalf of Gilead, Pfizer and MSD; he has also received research funds from Fondo de Investigación Sanitaria (FIS), Gilead, Scynexis, F2G and Cidara outside the submitted work. He currently acts as the scientific secretary of the EUCAST-AFST. M.C. has no speakers' bureaus or stock options to declare. J.B.L. is an employee and shareholder of Cidara Therapeutics, Inc. J.M. has, over the past 5 years, received research grants and honoraria from Gilead and Pfizer. O.Z. has, over the past 5 years, received research grants/contract work from Pfizer, Cidara and Gilead.

Supplementary data

Tables S1 and S2 is available as Supplementary data at *JAC* online.

References

1 Ong V, Hough G, Schlosser M *et al.* Preclinical evaluation of the stability, safety, and efficacy of CD101, a novel echinocandin. *Antimicrob Agents Chemother* 2016; **60**: 6872–9. https://doi.org/10.1128/AAC.00701-16

2 James KD, Laudeman CP, Malkar NB *et al.* Structure-activity relationships of a series of echinocandins and the discovery of CD101, a highly stable and soluble echinocandin with distinctive pharmacokinetic properties. *Antimicrob Agents Chemother* 2017; **61**: 1–8. https://doi.org/10.1128/AAC.01541-16

3 Lakota EA, Bader JC, Ong V *et al.* Pharmacological basis of CD101 efficacy: exposure shape matters. *Antimicrob Agents Chemother* 2017; **61**: e00758–17. https://doi.org/10.1128/AAC.00758-17

4 Sandison T, Ong V, Lee J *et al.* Safety and pharmacokinetics of CD101 IV, a novel echinocandin, in healthy adults. *Antimicrob Agents Chemother* 2016; **61**: AAC.01627–16. https://doi.org/10.1128/AAC.01627-16

5 Thompson GR, Soriano A, Skoutelis A *et al.* Rezafungin versus caspofungin in a phase 2, randomized, double-blind study for the treatment of candidemia and invasive candidiasis: the STRIVE trial. *Clin Infect Dis* 2021; **73**: e3647–55. https://doi.org/10.1093/cid/ciaa1380

6 Helleberg M, Jørgensen KM, Hare RK *et al.* Rezafungin in vitro activity against contemporary Nordic clinical *Candida* isolates and *Candida auris* determined by the EUCAST reference method. *Antimicrob Agents Chemother* 2020; **64**: 1–10. https://doi.org/10.1128/AAC.02438-19

7 Boikov DA, Locke JB, James KD *et al.* In vitro activity of the novel echinocandin CD101 at pH 7 and 4 against *Candida* spp. Isolates from patients with vulvovaginal candidiasis. *J Antimicrob Chemother* 2017; **72**: 1355–1358. https://doi.org/10.1093/jac/dkx008

8 Arendrup MC, Perlin DS, Jensen RH *et al.* Differential in vivo activities of anidulafungin, caspofungin, and micafungin against *Candida glabrata* isolates with and without FKS resistance mutations. *Antimicrob Agents Chemother* 2012; **56**: 2435–42. https://doi.org/10.1128/AAC.06369-11

9 Carvalhaes CG, Klauer AL, Rhomberg PR *et al.* Evaluation of rezafungin provisional CLSI clinical breakpoints and epidemiological cutoff values tested against a Worldwide Collection of Contemporaneous Invasive Fungal Isolates (2019 to 2020). *J Clin Microbiol* 2022; **60**: e0244921. https://doi.org/10.1128/jcm.02449-21

10 CLSI. Subcommittee (SC) on Antifungal Susceptibility Tests. June 2021 Meeting Minutes. https://clsi.org/media/gvuivvig/2021_summer_afsc_agenda_summary_minutes.pdf.

11 Arendrup MC, Meletiadis J, Zaragoza O *et al.* Multicentre determination of rezafungin (CD101) susceptibility of *Candida* species by the EUCAST method. *Clin Microbiol Infect* 2018; **24**: 1200–4. https://doi.org/10.1016/j.cmi.2018.02.021

12 European Committee on Antimicrobial Susceptibility Testing. MIC distributions and Epidemiological Cut-Off Value (ECOFF) Setting, EUCAST SOP 10.2, 2021. http://www.eucast.org.

13 Arendrup MC, Jørgensen KM, Hare RK *et al.* EUCAST Reference testing of rezafungin susceptibility and impact of choice of plastic plates. *Antimicrob Agents Chemother* 2019; **63**: 1–9. https://doi.org/10.1128/AAC.00659-19

14 Arendrup MC, Jørgensen KM, Hanemaaijer N *et al.* ISO Standard 20776-1 or serial 2-fold dilution for antifungal susceptibility plate preparation: that is the question! *J Antimicrob Chemother* 2021; **76**: 1793–9. https://doi.org/10.1093/jac/dkab088

15 Kavanagh A, Ramu S, Gong Y *et al.* Effects of microplate type and broth additives on microdilution MIC susceptibility assays. *Antimicrob Agents Chemother* 2019; **63**: e01760-18. https://doi.org/10.1128/AAC.01760-18

16 Arhin FF, Sarmiento I, Belley A *et al*. Effect of polysorbate 80 on oritavancin binding to plastic surfaces: implications for susceptibility testing. *Antimicrob Agents Chemother* 2008; **52**: 1597–603. https://doi.org/10.1128/AAC.01513-07

17 Sader HS, Rhomberg PR, Flamm RK *et al*. Use of a surfactant (polysorbate 80) to improve MIC susceptibility testing results for polymyxin B and colistin. *Diagn Microbiol Infect Dis* 2012; **74**: 412–4. https://doi.org/10.1016/j.diagmicrobio.2012.08.025

18 Zuill DE, Almaguer AL, Arendrup MC *et al*. Development and validation of a modified EUCAST yeast broth microdilution MIC method for rezafungin to mitigate nonspecific binding through incorporation of Tween 20. *Eur Congr Clin Microbiol Infect Dis* 2021: S87.

19 Arendrup MC, Meletiadis J, Mouton JW *et al*. EUCAST technical note on isavuconazole breakpoints for *Aspergillus*, itraconazole breakpoints for *Candida* and updates for the antifungal susceptibility testing method documents. *Clin Microbiol Infect* 2016; **22**: 571.e1–4. https://doi.org/10.1016/j.cmi.2016.01.017

20 Jensen RH, Arendrup MC. *Candida palmioleophila*: characterization of a previously overlooked pathogen and its unique susceptibility profile in comparison with five related species. *J Clin Microbiol* 2011; **49**: 549–56. https://doi.org/10.1128/JCM.02071-10

21 Turnidge J, Kahlmeter G, Kronvall G. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clin Microbiol Infect* 2006; **12**: 418–25. https://doi.org/10.1111/j.1469-0691.2006.01377.x

22 Meletiadis J, Curfs-Breuker I, Meis JF *et al*. In vitro antifungal susceptibility testing of *Candida* isolates with the EUCAST methodology, a new method for ECOFF determination. *Antimicrob Agents Chemother* 2017; **61**: e02372–16. https://doi.org/10.1128/AAC.02372-16

23 Espinel-Ingroff A, Arendrup MC, Pfaller MA *et al*. Interlaboratory variability of caspofungin MICs for *Candida* spp. using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? *Antimicrob Agents Chemother* 2013; **57**: 5836–42. https://doi.org/10.1128/AAC.01519-13

24 Adeel A, Qu MD, Siddiqui E *et al*. Expanded access use of rezafungin for salvage therapy of invasive *Candida glabrata* infection: a case report. *Open Forum Infect Dis* 2021; **8**: ofab431. https://doi.org/10.1093/ofid/ofab431

25 Lepak AJ, Zhao M, VanScoy B *et al*. Pharmacodynamics of a long-acting echinocandin, CD101, in a neutropenic invasive-candidiasis murine model using an extended-interval dosing design. *Antimicrob Agents Chemother* 2018; **62**: e02154-17. https://doi.org/10.1128/AAC.02154-17

26 Arendrup MC, Mavridou E, Mortensen KL *et al*. Development of azole resistance in *Aspergillus fumigatus* during azole therapy associated with change in virulence. *PLoS ONE* 2010; **5**: e10080. https://doi.org/10.1371/journal.pone.0010080