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Wild-type MIC distributions and epidemiological cutoff values for 5-flucytosine and *Candida* species as determined by EUCAST broth microdilution

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Objectives: EUCAST has established clinical breakpoints and epidemiological cutoff values (ECOFFs) for *Candida* spp. However, limited data are available for 5-flucytosine (5-FC). We assessed the *in vitro* susceptibility of 5-FC against a large collection of clinical *Candida* species using EUCAST methodology and determined the associated ECOFFs.

Methods: A total of 5622 *Candida* isolates were collected from patients across the Netherlands between 2008 and 2024. 5-FC MICs were determined using the EUCAST microbroth dilution reference method. Furthermore, MICs were extracted from the EUCAST website. The MICs from this study and those extracted were used to determine ECOFFs and local ECOFFs (L-ECOFFs).

Results: 5-FC exhibited potent *in vitro* activity against *C. albicans*, *N. glabratus* and *C. parapsilosis*, while decreased susceptibility was observed for *C. tropicalis, Pichia species, K. marxianus, Y. lipolytica,* and *C. auris*. The ECOFFs (mg/L) and the percentages of WT isolates for 5-FC were: *C. albicans*: 0.5 (97.2%), *N. glabratus*: 0.5 (96.6%), *C. parapsilosis*: 0.5 (99.5%) and *P. kudriavzevii*: 8 (99.4%). The L-ECOFF (mg/L) and the percentages of WT isolates for 5-FC were: *C. dubliniensis*: 0.25 (96.8%), *C. tropicalis*: 0.25 (67.2%), *K. marxianus*: 0.25 (48.0%), *C. lusitaniae*: 0.25 (86.5%), *M. guillermondii*: 0.125 (95.9%) and *P. norvegiensis*: 8 (94.2%).

Conclusions: 5-FC remains a valuable drug to manage difficult-to-treat invasive *Candida* infections. *In vitro* susceptibility cannot be predicted based on species identification for most *Candida* species, but requires MIC-testing. ECOFFs will help to interpret the MICs to support treatment decisions.

Introduction

Candida species are responsible for a variety of infections, including superficial infections, such as oral and vaginal thrush, as well as more severe infections, such as bloodstream, abdominal, and deep-tissue infections.[1] They are the most important cause of opportunistic mycoses and mycosis-associated mortality worldwide.[2]

Current clinical practice guidelines recommend first-line treatment with echinocandins or a lipid formulation of amphotericin B, depending on the clinical presentation, for management of invasive or disseminated forms of candidiasis. Fluconazole, a triazole antifungal, is extensively used to treat (muco)cutaneous infections and as a step-down therapy for invasive candidiasis and candidemia.[3,4] The varying susceptibility of yeast species to antifungal agents, along with the presence of intrinsic and secondary resistance, highlights the importance of antifungal susceptibility testing.

5-flucytosine (5-FC) was first introduced in 1968 as an antifungal agent for cryptococcosis and candidiasis treatment.[5] However, due to high toxicity and rapid resistance development when used alone, it is now mainly used in combination with amphotericin B for cryptococcal meningoencephalitis treatment.[6] Despite these limitations, 5-FC may still play a role in treating difficult-to-treat infections such as *Candida* endocarditis, meningitis, or endophthalmitis with fluconazole-resistant isolates.[3]

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In certain cases, it can also be used as a monotherapy for lower urinary tract infections caused by fluconazole-resistant *Nakaseomyces glabratus* (formerly *Candida glabrata*).[3]

The emergence of resistance to first- and second-line treatments, including multi-drug resistance in *C. auris*,[7] fluconazole and pan-azole resistant *C. tropicalis*,[8] and fluconazole-resistant *C. parapsilosis*,[9] along with slow progress in developing new antifungal agents, has resulted in a narrowing range of therapeutic options. Consequently, the likelihood of requiring combination antifungal therapy involving 5-FC is higher for the treatment of difficult-to-treat *Candida* infections.

Several studies have investigated the susceptibility of yeast to 5-FC using CLSI reference methods for antifungal susceptibility testing.[10,11] Contemporary data on 5-FC susceptibility in *Candida* species using EUCAST reference methods are limited. Moreover, the absence of EUCAST clinical breakpoints (CBPs) and epidemiological cut-off values (For EUCAST ECOFFs or for CLSI ECVs) for 5-FC and *Candida* species limits the interpretation of 5-FC MICs. To address this, we sought to describe the 5-FC EUCAST MIC distribution of *Candida* species, establish ECOFFs, and determine the number of non-WT isolates.

Material and methods

Isolates

From 2008 to 2024, 7231 clinical *Candida* isolates obtained from different anatomical sites were cultured in or submitted to the Radboudumc-CWZ Center of Expertise for Mycology for antifungal susceptibility testing. Isolates were identified using Bruker Biotyper MALDI-TOF MS. The following species were included in the study; *C. albicans* (2902 isolates), *Nakaseomyces glabratus (*1281) *C. parapsilosis* (361), *C. metapsilosis* (22), *C. orthopsilosis* (2), *C. tropicalis* (359), *Pichia kudriavzevii* (previously *C. krusei*) (229), *C. dubliniensis* (131), *Kluyveromyces marxianus* (previously *C. kefyr*) (83), *Clavispora lusitaniae* (*C. lusitaniae)* (74), *Meyerozyma guilliermondii* (*C. guilliermondii)* (66), *Pichia norvegensis* (*C. norvegensis*) (60), *C. auris* (14) and *Yarrowia lipolytica* (*C. lipolytica)* (13), *Pichia cactophila (C. inconspicua)* (12), *Diutina rugosa* (*C. rugosa*) (7), *Debaryomyces hansenii (C. famata)* (7)) (Table 1).

Susceptibility testing

A standard antifungal powder of 5-FC was obtained from Sigma (St. Louis, Mo.). Antifungal susceptibility testing followed the EUCAST microbroth dilution reference method.[12] The absorbance was measured at 405 nm using a spectrophotometer (Anthos Labtec Instruments GmbH, Salzburg, Austria). Quality control was ensured by testing the EUCAST-recommended strains, *C. parapsilosis* ATCC 22019 and *P. kudriavzevii* ATCC 6258. The MIC ranges, modal MIC (most common MIC), MIC_{50} (MIC that inhibits 50% of the isolates), and MIC_{90} (MIC that inhibits 90% of the isolates) were calculated.

ECOFFs, local ECOFFs and determining

ECCOF is defined as the upper MIC value at which the WT distribution ends. We calculated the EUCAST ECOFFs (EUCAST_ECOFFs) for 5-FC for aggregated EUCAST MIC distributions (extracted from the EUCAST website (http://mic.eucast.org/Eucast2/, data retrieved on: 1-11-2023) for the species that meet the requirements for ECOFF determination as set out by EUCAST recommendations and previously described in the formal EUCAST SOP 10.2 (MIC distributions and the setting of epidemiological cut-off values, https://www.eucast.org/eucastsops). In addition, local ECOFFs (L-ECOFFs) for local MIC distributions (yeast strains isolated from various hospitals in the Netherlands were included, but susceptibility testing was performed in a single laboratory). Among the several methods available to determine ECOFFs; we used four of these methods for comparison purposes the visual inspection,[13] the statistical method using ECOFFinder program,[14] The $MIC_{50}+2$ method,[15] and mode + 2 method.[16] The available literature describing 5-FC epidemiological cut off values ECVs for *Candida* species determined for other antifungal susceptibility methods ECVs such as CLSI[10] and Sensititre Yeast One (SYO)[11] were retrieved and those ECVs were extracted for comparison.

Non-wildtype (NWT) isolates

The WT and NWT isolates in this study were determined for individual species. To determine NWT isolates, we used EUCAST-ECOFFs following the EUCAST- recommendation taking into account only the visual inspection and the statistical ECOFFs (97.5%). In the case of the aggregated MIC distribution didn't meet the requirement for formal ECOFFs determination as described previously (truncated, small) we used the L-ECOFFs determined with the visual inspection methods. Additionally, we calculated the rate of isolates with high MIC values (>16 mg/L), which was proposed as the clinical breakpoint for *C. albicans*.[17]

Results

Susceptibility data

Table 1 and Figure 1 present the local 5-FC MIC distributions. The MIC values of the quality control strains were within the acceptable limits. 5-FC displayed a species-specific *in vitro* activity with the lowest MIC90 (MIC90, ≤0.25 mg/L) observed against *C. parapsilosis*, *N. glabratus*, *C. dubliniensis, C. albicans* and *M. guillermondii*. Higher MIC values were observed for *C. lusitaniae* (MIC90, 1 mg/L), *P. kudriavzevii* (MIC90, 4 mg/L), *P. norvegiensis MIC90,* (8 mg/L), *K. marxianus* (MIC90, 8 mg/L) *and C. tropicalis* (MIC90, 64 mg/L). Most of the MIC distributions were unimodal except for *C. tropicalis and K. marxianus* where the distribution where bimodal and multimodal, respectively. Table 2 summarizes the aggregated MIC data extracted from the EUCAST website. Table 2 summarizes the aggregated MIC data extracted from the EUCAST website. Overall, the local MIC distributions were one to two 2-fold dilutions lower than the aggregated EUCAST MIC distributions based on MIC_{50} and modal MIC exept for 2 species. For *M. guillermondii* the local MIC50 and local modal MIC were ≤0.03 mg/l ≤ 0.03 mg/L while the aggregated MIC50 and modal MIC were 0.25 mg/L and 0.125 mg/L, representing a span of at least two 2-fold dilutions. For *K. marxianus,* the local modal MIC was 0.06 mg/L while the aggregated modal MIC was 1 mg/L.

ECOFFs determination and wild-type populations

Table 3 presents the ECOFFs calculated using ECOFFinder and the $MIC_{50}+2$ mode MIC + 2 methods for both local MIC distributions

Table 1. Local 5-Fluorocytosine MIC distributions of 5622 clinical Candida species isolates determined by EUCAST broth microdilution

	5-Fluorocytosine MIC (mg/L)														
Species	≤ 0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	> 64	Total	Range
Candida albicans	627	817	**969**	241	163	38	9	18	9	1	1	4	5	2902	≤ 0.03–>64
Candida dubliniensis	51	**61**	12	4	1	0	0	0	0	0	0	0	2	131	≤ 0.03–>64
Nakaseomyces glabratus	504	**542**	179	8	3	6	14	14	1	2	2		6	1281	≤ 0.03–>64
Candida parapsilosis	93	**151**	94	16	4	1				1			1	361	≤ 0.03–>64
Candida metapsilosis	6	**16**												22	≤ 0.03–0.25
Candida orthopsilosis		**2**												2	0.06
Candida tropicalis	85	**95**	48	6	3			3	3	10	64	39	3	359	≤ 0.03–>64
Pichia kudriavzevii					2	31	**93**	81	19	2			1	229	0.5–>64
Kluyveromyces marxianus	13	17	3	1	4	7	4	12	**20**	1	1	0	0	83	≤ 0.03–32
Clavispora lusitaniae	**35**	26	3	1	1	2	2	1	0	1	2	0	0	74	≤ 0.03–32
Meyerozyma guillermondii	**41**	18	4	2	0	0	1	0	0	0	0	0	0	66	≤ 0.03–2
Pichia norvegiensis	2				1	7	**23**	15	8	4				60	≤ 0.03–16
Pichia cactophila	1				3	**7**	1							12	≤ 0.03–2
Candida auris	0	5	**7**										2	14	≤ 0.03–>64
Duitina rugosa	**3**		2	2										7	≤ 0.03–0.25
Debaryomyces hansenii	1		**3**	1	2									7	≤ 0.03–0.5
Yarrowia lipolytica	0	1	1	1	1	1	**3**	0	1	0	0	0	4	13	0.06–>64
Total	1462	1751	1325	283	188	100	150	144	61	22	69	43	24	5622	

MIC_{50}, MIC that inhibits 50% of the isolates; MIC_{90}, MIC that inhibits 90% of the isolates; Modal MIC, the most common MIC are highlighted in bold, marked in gray and underlined (respectively).

and aggregated EUCAST MIC distributions. In addition, the ECVs published by Pfaller *et al*. for CLSI[10] and by Cantón *et al*. for SYO[11] were included for comparison.

Among the aggregated EUCAST MIC distributions, only *C. albicans*, *N. glabratus*, *C. parapsilosis* and *P. kudriavzevii* distributions met the criteria for EUCAST_ECOFFs (distribution must be collected from at least five centre, number of isolates per species >100 and the distribution should not be truncated).[18] For the other species formal EUCAST_ECOFFs determination was not possible due to insufficient isolates number (*C. lusitaniae* and *K. marxianus*) or inadequate truncated distributions (*C. tropicalis* and *M. guillermondii)*.

L-ECOFF values were determined using our local distributions for eight species with >50 MICs using ECOFFinder and mode+2 and MIC50+2 methods. For *P. cactophila*, *C. auris*, *D. rugosa*, *D. hansenii* and *Y. lipolytica*, the number of individual MICs was too low for L-ECOFF calculation and *C. lusitaniaea* and *M. guillermondii* the distributions were truncated and L-ECOFFs were only established using visual inspection.

For the identification of the final ECOFFs for 5-FC to distinguish the WT-population and NWT population, we considered the ECOFFs 97.5% calculated based on the methods of Turnidge *et al*. for the species that did meet the requirement from the aggregated data following the recommendation of the formal EUCAST SOP 10.2. The following ECOFFs (percentage of WT-population) were identified: 0.5 mg/L (97.1%), 0.5 (96.5%), 0.5 (99.2%) and 8 (98.7%) for *C. albicans, N. glabratus, C. parapsilosis and P. kudriavzevii,* respectively.

For the other species the L-ECOFFs based on visual inspection were used to identify the WT population the following L-ECOFFs (percentage of the WT-population) were established: 0.25 mg/L (97.7%) for *C. dubliniensis*, 0.25 (66.2%) *for C. tropicalis,* 0.5 (45.8%) for *K. marxianus,* 0.25 mg/L (87.8%) for *C. lusitaniae*, 0.25 (98.5%) for *M. guillermondii*, 8 (93.33%) for *P. norvegiensis.*

Generally, the ECOFFs determined using the MIC_{50}+2 method were identical or one 2-fold dilution higher than the ECOFFs 97.5% calculated using ECOFFinder. The mode+2 and MIC_{50}+2 were within 1 dilution for both local and EUCAST_ECOFFs, except for *K. marxianus* L_ECOFF, where mode+2 was 0.25 mg/L, while MIC_{50}+2 was 2 mg/L. Furthermore, EUCAST_ECOFFs (97.5%) were similar to L-ECOFFs (97.5%) for *C. albicans* and *P. kudriavezii,* one 2-folds dilution higher for *C. parapsilosis* and two 2-fold dilutions higher for *N. glabratus.* L_ECOFFs mode+2 and MIC_{50}+2 were 1-2 2-fold dilutions lower than the corresponding mode+2 and MIC_{50}+2 EUCAST_ECOFFs.

NWT isolates and isolates with MIC >16 mg/L

Overall, 196 isolates out of 5622 (349%) were classified as 5-FC NWT. The proportion of NWT isolates was below 5% for *C. parapsilosis* (0.8%), *N. glabratus* (3.5%), *C. dubliniensis* (1.5%) *P. kudriavzevii* (1.3%), *M. guillermodii* (1,5%), *C. albicans* (2.9%). In contrast, the proportion of NWT isolates exceeded 25% in *C. tropicalis* (34.0%) and *K. marxianus* (53.6%) (Table 4).

The proportion of isolates with MIC >16 mg/L was below 2% for most species, except for C. *lusiataniae* (2.7%), *C. auris* (14.3%), *Y. lipolytica* (30.8%) and *C. tropicalis* (29,5%). In total, 137 isolates had an MIC >16 mg/L. The majority of these isolates (106 isolates 77.4%) belonged to *C. tropicalis*, followed by *C. albicans* (10 isolates, 7.3%) and *N. glabratus* (8 isolates, 5.8%),

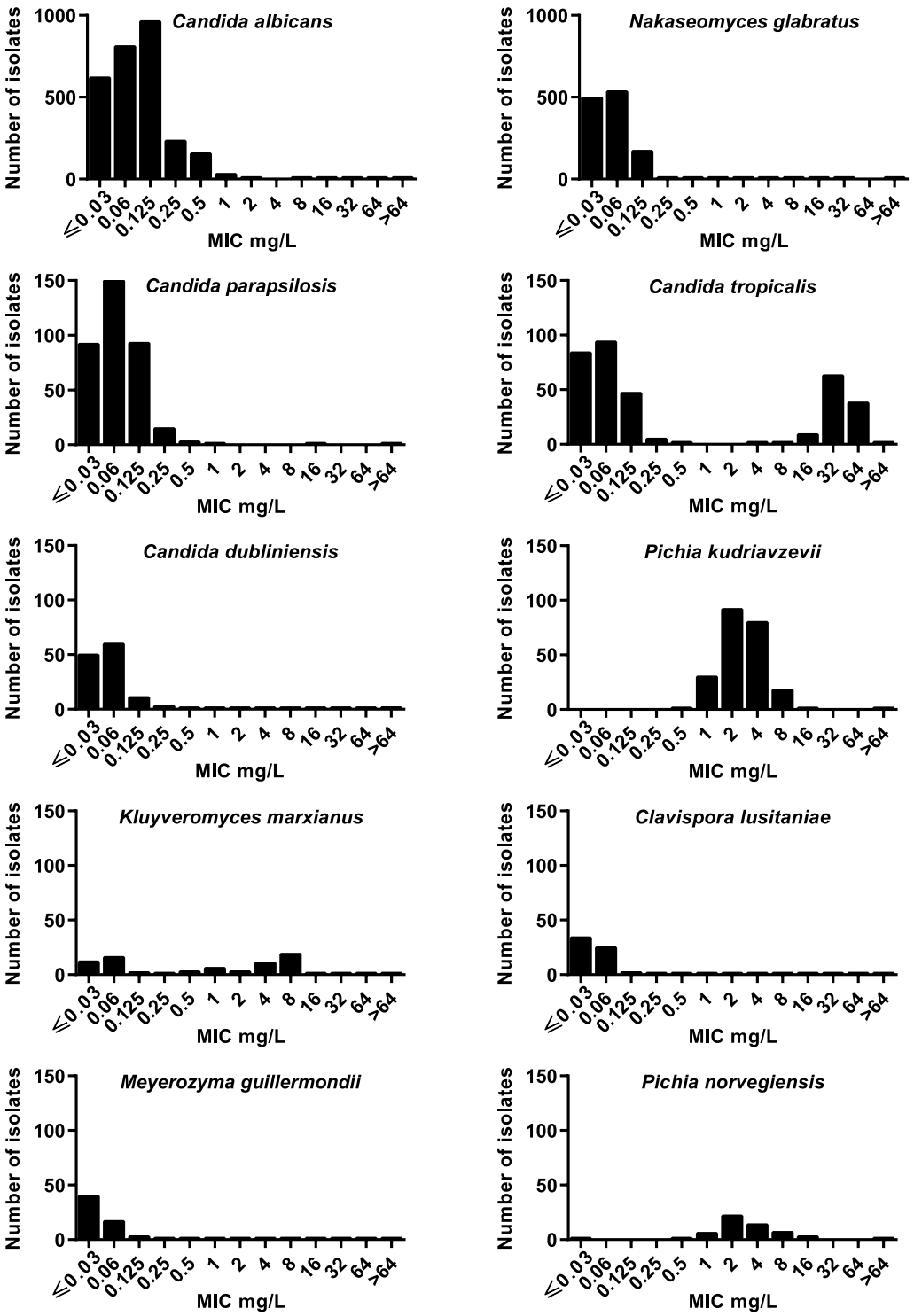

Figure 1. 5-Fluorocytosine MICs distribution of C. albicans, N. glabratus, C. parapsilosis, C. tropicalis, C. dubliniensis, P. kudriavzevii, K. marxianus, C. lusitaniae, M. guillermondii and P. norvegensis. Note that the X-axis ranges from 0–1000 for C. albicans and N. glabratus but from 0–150 for all other species.

Y. lipolytica (4 isolates, 2.9%) and *C. auris*, *C. lusitaniae* and *C. dubliensis* (2 isolates, 1.5%). For *C. auris* 13 of 15 isolates had MIC <0.25 mg/l and two MIC >64 mg/L while for *Y. lipolytica* the isolates are distributed in higher MICs: four isolates <0.5 mg/L), one isolate (1 mg/L), four isolates (2 mg/L), one (8 mg/L) and four isolates (>64 mg/L).

Table 2. Aggregated EUCAST 5-FCMIC distributions

Species	5-Fluorocytosine MIC (mg/L)												Distributions	Total	Range
	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128			
Candida albicans	89	693	**734**	206	78	33	25	27	19	1	4	32	10	1941	0.06–128
Candida famata (Debaryomyces hansenii)	0	3	**21**	5	2	0	2	0	1	0	1	0	8	35	0.125–64
Candida glabrata (Nakaseomyces glabratus)	29	150	**192**	9	6	2	3	11	9	0	1	5	10	417	0.06–128
Candida guillermondii (Meyerozyma guilliermondii)	0	**28**	19	4	2	1	1	8	2	0	0	0	8	65	0.125–16
Candida kefyr (Kluyveromyces marxianus)	1	1	2	2	**5**	1	3	3	0	0	0	0	6	18	1–8
Candida krusei (Pichia kudriavzevii)	0	0	0	4	8	66	**88**	18	25	1	0	1	10	211	0.5–128
Candida lusitaniae (Clavispora lusitaniae)	5	**20**	13	1	0	0	0	0	2	1	3	1	10	46	0.06–128
Candida parapsilosis	31	192	**261**	66	49	9	5	0	1	0	1	2	10	617	0.06–128
Candida tropicalis	0	**149**	144	18	4	4	2	3	9	5	7	0	9	345	0.125–64

MIC_{50}, MIC that inhibits 50% of the isolates; MIC_{90}, MIC that inhibits 90% of the isolates; Modal MIC, the most common MIC are highlighted in bold, marked in gray and underlined (respectively).

Discussion

Effective management of invasive candidiasis requires knowledge of the susceptibility patterns of various *Candida* species to antifungal drugs. While 5-FC is an old antifungal drug, limited studies have described its MIC distributions using the EUCAST methodology. Moreover, the absence of EUCAST ECOFFs and CBPs for interpreting 5-FC susceptibility complicates treatment decisions for clinicians.

While there are CBPs and ECVs for 5-FC and some *Candida* species according to CLSI, CLSI ECVs, and SOY ECVs,[10,11] they cannot be directly applied to EUCAST assays due to differences in technical aspects.[15] Although a study showed perfect essential agreement (EA) and categorical agreement (CA) between CLSI and EUCAST for 5-FC, except for *C. krusei* (EA, 94%), and *C. albicans* (CA, 88%). However, only a limited number of species (*C. albicans, C. glabrata, C. parapsilosis*, *C. tropicalis* and *C. krusei)* were examined in this study.[19]

We analysed the MIC distributions of various yeast species using local and pooled EUCAST MIC data aiming to determine ECOFFs for differentiating between WT and NWT isolates. Several methods have been reported for determining ECOFFs, including those by Arendrup *et al.*[15] who estimated $MIC_{50}+2$ ECOFFs as two 2-fold dilution steps higher than the MIC_{50}, Rodríguez-Tudela *et al*. estimated them as two 2-fold dilutions above the modal MIC,[16] and a statistical program was developed to calculate the ECOFFs.[14] To formally establish ECOFFs, at least five datasets, of at least 15 MICs, totaling 100 MICs, and with the modal MIC within ±1 twofold dilution from the most common modal MIC are required.[20] We extracted data from the EUCAST website to determine the ECOFF. Four species had >100 MICs and were from more than five datasets, thus fulfilling the requirements. However, as this is an aggregated dataset, it is unknown whether all datasets consisted of at least 15 MICs and whether the modal MIC was within ±1 two-fold dilution of the most common modal MIC. As the statistical model could not be used for most MIC distributions, we also calculated the ECOFFs using two alternative methods: the $MIC_{50}+2$ and mode + 2 methods.[15,16]

CPBs are currently not available for EUCAST 5-FC MICs. However, Hope *et al.*[17] demonstrated that *C. albicans* isolates with MICs of up to 16 mg/L could still be treated with a human dose of 100 mg/kg/d of 5-FC. This conclusion was based on the results of an *in vivo* mouse model and Monte Carlo simulations of human 5-FC dosing, which showed over 95% target attainment for optimal 5-FC activity at a dose of 100 mg/kg/day for isolates with a 5-FC MIC of 16 mg/L, while the simulated target attainment was below 95% for isolates with an MIC of 32 mg/L.[17] Based on this, the authors proposed a EUCAST 5-FC CPB of >16 mg/L for *C. albicans.*[17] This CPB is in agreement with the CLSI CPB of ≥32 mg/L, which has often been employed in previous studies.[10,11] Although no *in vivo* data were available for other species, we also applied the proposed EUCAST CPB to the analysis of other species.

The susceptibility of 5-FC to *Candida* species varies depending on the species and geographical region.[21] It has been shown to have potent *in vitro* activity against *C. albicans*, *N. glabratus* and *C. parapsilosis*, which is consistent with previous studies that used the CLSI methodology.[22–24] However, there are some discrepancies in the MIC values reported for *C. albicans*, Some studies have found higher MIC values for 5-FC in *C. albicans* isolates from North America and Africa, which is associated with the high prevalence of serotype B in these countries.[25,26] The A101C mutation in the FUR1 gene has been linked to increased MICs in this serotype.[27] Additionally, a clade of *C. dubliniensis* with increased 5-FC MICs has been found exclusively in Middle Eastern countries.[28] However, other studies have reported higher MIC values for 5-FC in *N. glabratus* isolates from Italy and Spain.[25,29] In our study, we did not determine the clades or serotypes of the *Candida* strains, so we could not determine whether the differences in MIC values were due to differences in the clades. We did separately report the MIC values of *C. parapsilosis*

Table 3. Comparison of ECOFFs obtained for 5-FC by use of different methods

	L-ECOFFs								EUCAST_ECOFFs								Final EUCAST ECOFFs (L-ECOFFs)	CLSI ECVs	Sensititre ECVs
	visual inspection	ECOFF 95.0%	ECOFF 97.5%	ECOFF 99.0%	ECOFF 99.5%	ECOFF 99.9%	mode +2	MIC_{50}+2	visual inspection	ECOFF 95.0%	ECOFF 97.5%	ECOFF 99.0%	ECOFF 99.5%	ECOFF 99.9%	mode +2	MIC_{50}+2			
Candida albicans	0.5	0.25	0.5	0.5	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	1	1	1	0.5	0.5	1
Candida dubliniensis	0.25	0.125	0.125	0.125	0.125	0.25	0.25	0.25									(0.25)	0.5	
Nakaseomyces glabratus	0.25	0.125	0.125	0.125	0.25	0.25	0.25	0.25	0.5	0.5	0.5	0.5	0.5	0.5	1	1	0.5	0.5	0.25
Candida parapsilosis	0.25	0.125	0.25	0.25	0.25	0.5	0.25	0.25	0.5	0.5	0.5	0.5	1	1	1	1	0.5	0.5	0.5
Candida tropicalis	0.5	0.125	0.125	0.25	0.25	0.5	0.25	0.25										0.5	0.5
Pichia kudriavzevii	8	8	8	8	8	16	8	8	8	8	8	8	8	16	16	16	8	32	32
Kluyveromyces marxianus	0.5	0.12	0.125	0.125	0.125	0.125	0.25	2									(0.5)		
Clavispora lusitaniae	0.25																(0.25)	0.5	
Meyerozyma guillermondii	0.25																(0.25)	1	
Pichia norvegiensis	8	8	8	16	16	32	8	8									(8)		
Pichia cactophila																			
Candida auris																			
Duitina rugosa																			
Debaryomyces hansenii																			
Yarrowia lipolytica																			

L_ECOFF: local epidemiolocal cutoff value. EUCAST_ECOFFs ECOFF of aggregated EUCAST MIC data retrieved from the EUCAST MIC website. ECOFF 95.0%–99.9%: ECOFFs calculated with ECOFFinder. Mode+2: ECOFF was determined using the mode MIC+2 dilutions. MIC_{50}+2: ECOFF determined by taking the MIC_{50}+2 dilution. CLSI ECV (epidemiological cutoff values) from[10] and Sensititre Yeast One from.[11]

Table 4. Percentage of WT, NWT and isolates with MIC >16 mg/L of 5622 Candida isolates

Organism	WT (%)	NWT (%)	MIC >16 mg/L (%)
Candida albicans	2817 (97.1)	85 (2.9)	10 (0.3)
Candida dubliniensis	128 (97.7)	3 (2.3)	2 (1.5)
Nakaseomyces glabratus	1236 (96.5)	45 (3.5)	8 (0.6)
Candida parapsilosis	358 (99.2)	3 (0.8)	1 (0.3)
Candida metapsilosis	N/A	N/A	0
Candida orthopsilosis	N/A	N/A	0
Candida tropicalis	237 (66.0)	122 (34.0)	106 (29.5)
Pichia kudriavzevii	226 (98.7)	3 (1.3)	1 (0.4)
Kluyveromyces marxianus	38 (45.8)	45 (54.2)	
Clavispora lusitaniae	65 (87.8)	9 (12.2))	2 (2.7)
Meyerozyma guillermondii	65 (98.5)	1(1.5)	0
Pichia norvegiensis	56 (93.3)	4 (6.7)	0
Pichia cactophila	N/A	N/A	0
Duitina rugosa	N/A	N/A	0
Debaryomyces hansenii	N/A	N/A	0
Candida auris	N/A	N/A	2 (14.3)
Yarrowia lipolytica	N/A	N/A	4 (30.8)
Total			137 (2.4)

N/A, non-applicable (no ECOFF available); NWT, non-wild type. NWT was calculated with the local ECOFF (97.5%).

sensu strictu (referred to *C. parapsilosis* in this study) and *C. orthopsilosis* and C. *metapsilosis* as MIC values may differ within species complexes. However, the number of tested isolates of *C. orthopsilosis* and C. *metapsilosis* was too low to draw strong conclusions.

In our study, 5-FC exhibited low MICs against *M. guillermondii* and *D. rugosa*. The low MIC values observed for *M. guillermondii* in our study are in contrast to the elevated MIC values reported in previous studies using CLSI broth microdilution, where MIC_{90} values of 4 mg/L and 16 mg/L were documented.[22,30] In addition, the local MIC distribution for *M. guillermondii* was two 2-fold dilution steps lower than the aggregated EUCAST dataset. Whether this discrepancy can be attributed to methodological differences or reflects a genuine distinction in susceptibility between collections remains unclear.

We observed high 5-FC MICs for *Pichia* species, *C. lusitaniae, C. auris, K. marxianus and Y. lipolytica*. Previous studies indicate that *Pichia* species are characterized by elevated 5-FC and fluconazole MICs.[30] However, unlike other studies where the MIC_{90} was >32 mg/L and isolates with 5-FC MIC >16 mg/L in *P. kudriavzevii* were relatively high, ranging from (1.9% to 4%),[23] in our study, the MIC_{90} was 8 mg/L and only 0.6% of the isolates had a MIC >16 mg/L.

Candida auris, a recently emerging pathogen, has caused challenging nosocomial outbreaks globally and frequently exhibits resistance to fluconazole and voriconazole.[7] Szekely *et al*., reported that 8 in 62 (13.1%) *C. auris* isolates had a MIC >16 mg/L, primarily in the southern Asian clade.[31] This study included isolates recovered from the urinary tract from patients that were actively treated with 5-FC and may thus reflect acquired 5-FC resistance.[7] In another study no *C. auris* isolates with 5-FC MIC >16 mg/L were found in 400 tested isolates.[32] 5-FC The limited inclusion of *C. auris* isolates in this study precluded strong conclusions regarding susceptibility patterns in *C. auris*.

C. lusitaniae is a rare pathogen known for severe and potential fatal opportunistic infections.[33,34] In our study the 5-FC MIC_{90} was 0.5 mg/L and three of the 74 isolates (4.1%) exhibited an MIC >16 mg/L. These findings align with most previous reports, which documented a 5FC MIC >16 mg/L range of 0%–9% for *C. lusitaniae*.[23,24,35] However, 19.4% of 186 isolates from the UK displayed MICs >16 mg/L with a MIC_{90} > 64 mg/L.[30]

We found a high proportion of NWT population *in C. tropicalis* (34.0%). Our findings showed that the rate of isolates with MIC >16 mg/L was high for *C. tropicalis* (29.5%) compared to other species, such as *C. albicans* (0.3%), *C. parapsilosis* (0.3%), *P. kudriavzevii* (0.4%), *N. glabratus* (0.6%), and *C. dubliniensis* (1.5%). *C. tropicalis* is the second most virulent *Candida species* after *C. albicans* and has been reported as the first cause of candidemia in some countries. This yeast is commonly isolated from immunocompromised patients and is associated with the poorest prognosis and high mortality among *Candida* species. It is able to develop resistance to routine antifungal drugs such as fluconazole.[36] Previous studies have reported varying resistance rates and MIC profiles for *C. tropicalis* against 5-FC. While some studies have found lower MIC profiles and full susceptibility,[12,13,16] others have reported higher MIC values and resistance rates ranging from 7%–58.3%.[23–25,37,38] The MIC distribution of *C. tropicalis* against 5-FC was bimodal consistent with a previous report from France describing a 5-FC resistant clone[37] and Germany were high rates of resistance were described.[38]

K. marxianus displayed a multimodal distribution, with over half of the isolates being NWT and distributed in higher MICs. This suggested the presence of multiple resistance mechanisms in the species. Borman *et al*. found a high MIC90 of 4 mg/L but a unimodal distribution.[30] In contrast, Pfaller *et al*. found 15 *K. marxianus* isolates with an MIC below 2 mg/L using the CLSI method.[23]

The same pattern was observed in *Y. lipolytica*. previous research also reported higher 5-FC MICs values for this species.[30,39,40] Desnos-Ollivier,[39] found a range of [1–64 mg/L] for 34 *Y. lipolytica* isolates and MIC 90 was ≥ 64 mg/L using the EUCAST method. The rates of clinical and environmental isolates with MIC values ≥ 32 mg/L were 11 out of 20 (55%) and 10 out of 13 (76.92%), respectively. Similarly, Yu et a found considerable variation in the MIC for 5-FC using CLSI, ranging from 0.25 µg/mL to 64 µg/mL, with L-ECOFFs of 8 mg/L for the CLSI.[40] Commercial methods yielded higher L-ECOFFs: 64, 128, and 256 mg/L using the ATB FUNGUS 3 (ATB), MIC test strip (MTS), and SYO, respectively.

The L_ECOFFs values found in this study were generally lower than the EUCAST_ECOFFs values. We observed that the local mode, MIC50, and MIC90 were typically one dilution lower than the EUCAST mode MIC50 and MIC90, resulting in a one dilution step lower L_ECOFF. For *C. lusitaniea* and *M. guillermondii*, the L_ECOFFs were two steps lower than EUCAST_ECOFF. These isolates had relatively low MICs in our study. As EUCAST only accepts non-truncated MIC distributions, and the 5-FC susceptibility testing range is commonly 0.06–64 mg/L, there may be a small bias towards laboratories with relatively high MICs. However, we used a distribution of 0.03–64 mg/L. Our L_ECOFFs and

ECUAST_ECOFFs values were similar to CLSI and SYO ECVs, except for *P. kudriavzevii*, where the ECOFF was two dilution lower than CLSI and ECVs (32 mg/L).[10,11]

We did not study the 5-FC resistance mechanism in this study. Many different genes and mutations are involved in 5-FC resistance and different mutations may have various effects.[6] Previous research has shown that isolates with lower or equal MIC values of 0.5 mg/L are wild-type and do not have any mutations, while those with MIC values between 0.5 and 16 mg/L have a mutation in FCY2, and those with MIC ≥ 32 mg/L have mutations in FCY1 and FUR1 genes.[41] The finding of a wide range of MICs for NWT isolates suggests that there is not a single resistance mechanism, but multiple mechanisms with different effects on the 5-FC susceptibility phenotype.

Some limitations of the study should be noted. For invasive yeast infections, 5-FC is always administered in combination with other antifungal agents. The 5-FC ECOFFs and CBPs are based on single-drug *in vitro* studies, and it is not known whether optimal drug exposure targets for 5-FC are similar in combination therapy. Additionally, isolates were sent from several centers for susceptibility testing to our reference lab and therefore likely contains a higher proportion of isolates with NWT phenotypes. The isolates were collected from various body sites, including blood, sterile body sites, and superficial sites. Consequently, not all isolates were from patients with *Candida* infections; some were from patients with mere colonization. Clinical history, including previous 5-FC and other antifungal drug exposure, is also unknown, making it impossible to determine whether observed NWT phenotypes are related to prior drug exposure. Furthermore, for species with fewer than 50 isolates, strong conclusions about the MIC distributions could not be established.

Our data contributes to understanding EUCAST 5-FC resistance in *Candida* species and aids in identifying NWT isolates with potential resistance mechanisms. More EUCAST MIC distribution data from other labs is needed to establish formal EUCAST ECOFFs.

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