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In Vitro Antifungal Susceptibility Testing of *Candida* Isolates with the EUCAST Methodology, a New Method for ECOFF Determination

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ABSTRACT The *in vitro* susceptibilities of 1,099 molecularly identified clinical *Candida* isolates against 8 antifungal drugs were determined using the EUCAST microdilution method. A new simple, objective, and mathematically solid method for determining epidemiological cutoff values (ECOFFs) was developed by derivatizing the MIC distribution and determining the derivatized ECOFF (dECOFF) as the highest MIC with the maximum second derivative. The dECOFFs were similar (95% agreement within 1 dilution) to the EUCAST ECOFFs. Overall, low non-wild-type/resistance rates were found. The highest rates were found for azoles with *C. parapsilosis* (2.7 to 9.8%), *C. albicans* (7%), and *C. glabrata* (1.7 to 2.3%) and for echinocandins with *C. krusei* (3.3%), *C. albicans* (1%), and *C. tropicalis* (1.7%).

KEYWORDS *Candida*, EUCAST, antifungal susceptibility testing, epidemiological cutoff value

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Although epidemiological cutoff values (ECOFFs) are not clinical breakpoints, they are useful and necessary for defining wild-type populations and detecting strains with MIC values outside the wild-type distribution that may reflect strains with reduced susceptibility (1, 2). However, for some antifungal drugs and *Candida* species, no ECOFFs hinder the detection of non-wild-type isolates. Determination of an ECOFF may be quite challenging, since it requires complex statistical analysis (3), and so far, no clear consensus on the best method has been reached. The CLSI is using a statistical approach whereby the log-normal distribution is iteratively fitted to different MIC subsets until the best fit is found (2). However, a perfect fit may not be attained, particularly for small MIC data sets or for nonsymmetric distributions or distributions with heavy tails. Truncated data cannot by analyzed because the normal distribution cannot be easily defined (3). Furthermore, the percentage of isolates that should be encompassed within the wild-type distribution is arbitrarily chosen to be between 95 and 99%, and it is strongly affected by the percentage of resistant isolates. Finally, different MIC subpopulations cannot be easily identified. For multimodal MIC distributions, a statistical approach was used to describe the MIC subpopulations of *Aspergillus fumigatus* and azoles (4). EUCAST utilized both a nonstatistical approach, namely, the "eyeball method," whereby ECOFFs are determined by visual inspection of the MIC distribution as the MIC at the beginning of the left tail, as well as the statistical approach mentioned above. Although with visual inspection one does not assume a specific shape of MIC distribution, it is subjective and difficult for nonsymmetrical distributions and MIC distributions with a high kurtosis.

TABLE 1 EUCAST clinical breakpoints and epidemiological cutoff values

Drug	Clinical breakpoints and ECOFFs for isolates from indicated strains (mg/liter)[a]														
	C. albicans			*C. parapsilosis*			*C. krusei*			*C. glabrata*			*C. tropicalis*		
	BP	ECOFF	dECOFF	BP	ECOFF	dECOFF	BP	ECOFF	dECOFF	BP	ECOFF	dECOFF	BP	ECOFF	dECOFF
Amphotericin B	1	1	2	1	1	2	1	1	2	1	1	2	1	1	2
Fluconazole	2/4	1	1	2/4	2	2	–[b]	128	16/256[d]	0.002/32	32	32	2/4	2	1
Itraconazole	0.06	0.06	0.03	0.12	0.12	0.06	IE[c]	1	1		2	2	0.12	0.12	0.06
Voriconazole	0.12	0.12	0.03	0.12	0.12	0.03	IE	1	1		1	1	0.12	0.12	0.06
Posaconazole	0.06	0.06	0.03	0.06	0.06	0.03	IE	0.5	0.5		1	1	0.06	0.06	0.06
Anidulafungin	0.03	0.03	0.06	0.002/4	4	4	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
Micafungin	0.016	0.016	0.03	0.002/4	2	4	IE	0.25	0.25	0.03	0.03	0.06	IE	0.06	0.06

[a]BP, breakpoint (S≤/R>). dECOFFs were determined in this study using the derivatization method applied to the MIC distributions on the EUCAST website.
[b]Susceptibility testing is not recommended, as the species is a poor target for therapy with the drug.
[c]Insufficient evidence to determine a breakpoint.
[d]The two maximum 2nd derivatives are at MIC 16 to 256 mg/liter and represent a resistant population.

Few large studies have used the EUCAST method to define wild-type (WT) distributions of antifungals and *Candida* spp. We therefore determined the susceptibilities of more than 1,000 molecularly identified clinical *Candida* sp. isolates to 8 antifungal drugs using the EUCAST methodology. Furthermore, a simple mathematical approach was developed for analyzing MIC distribution and for determining ECOFFs in an objective fashion.

We used 1,099 *Candida* strains collected from 871 patients recruited into clinical trials of invasive and esophageal candidiasis between 2002 and 2004 with no prior echinocandin exposure. Since WT distributions and ECOFFs are species specific, all strains were reidentified in our laboratory using molecular techniques, including amplified fragment length polymorphism (AFLP) and the internal transcribed spacer (ITS) technique, where appropriate (5). In total, 584 strains (53.1%) were identified as *C. albicans*, 180 (16.4%) as *C. tropicalis*, 122 (11.1%) as *C. parapsilosis*, 86 (7.8%) as *C. glabrata*, and 30 (2.7%) as *C. krusei*, as previously described (6). The remaining 97 (8.8%) isolates belong to other *Candida* spp. (i.e., *C. guilliermondii*, 20; *C. orthopsilosis*, 15; *C. lusitaniae*, 11; *C. dubliniensis*, 8; *C. rugosa*, 7; *C. kefyr*, 7; *C. pelliculosa*, 6; *C. fabiani*, 5; *C. lipolytica*, 3; *C. metapsilosis*, 3; *C. utilis*, 2; *C. fermentati*, 2; *C. intermedia*, 2; *C. inconspicua*, 1; *C. pararugosa*, 1; *C. famata*, 1; *C. palmioleophila*, 1; *C. xestobii*, 1; and *C. viswanathii*, 1) (6). No *C. dubliniensis* and no *C. glabrata* cryptic species were found, whereas within the *C. parapsilosis* complex ($n = 140$), 87.2% ($n = 122$) were *C. parapsilosis sensu stricto*, 10.7% ($n = 15$) were *C. orthopsilosis*, and 2.1% ($n = 3$) were *C. metapsilosis*. Microdilution testing was performed using the EUCAST E.DEF 7.3 method (7). The MIC distribution was constructed, and the MIC_{50} and MIC_{90} were calculated for each drug and species as the MICs that inhibit 50% and 90% of isolates, respectively. The percentage of resistant isolates was calculated using the EUCAST clinical breakpoints, when available, or ECOFFs when clinical breakpoints were not available (Table 1) (see http://www.eucast.org/clinical_breakpoints/).

In search of a new, more objective, and mathematically solid ECOFF determination method other than the eyeball method, we extracted MIC distributions for amphotericin B, fluconazole, voriconazole, posaconazole, itraconazole, anidulafungin, and micafungin from the EUCAST website (see http://mic.eucast.org/Eucast2/), and the dECOFF was determined for each MIC distribution by calculating the numerical second derivative at each MIC of the distribution (GraphPad Prism 4.0, San Diego, CA). The second derivative describes the change in steepness of the MIC distribution at each MIC. It becomes zero at inflection points where the curve changes from concave to convex, or vice versa (1 standard deviation [SD] away from the mean of a normal distribution), minimum at the point with the greatest concavity (center of a unimodal distribution), and maximum at points with the greatest convexity (~1.73 SDs away from the mean of a normal distribution). The area within the maximum second derivatives around the mean contains most of the data and can be used to define the wild-type population,

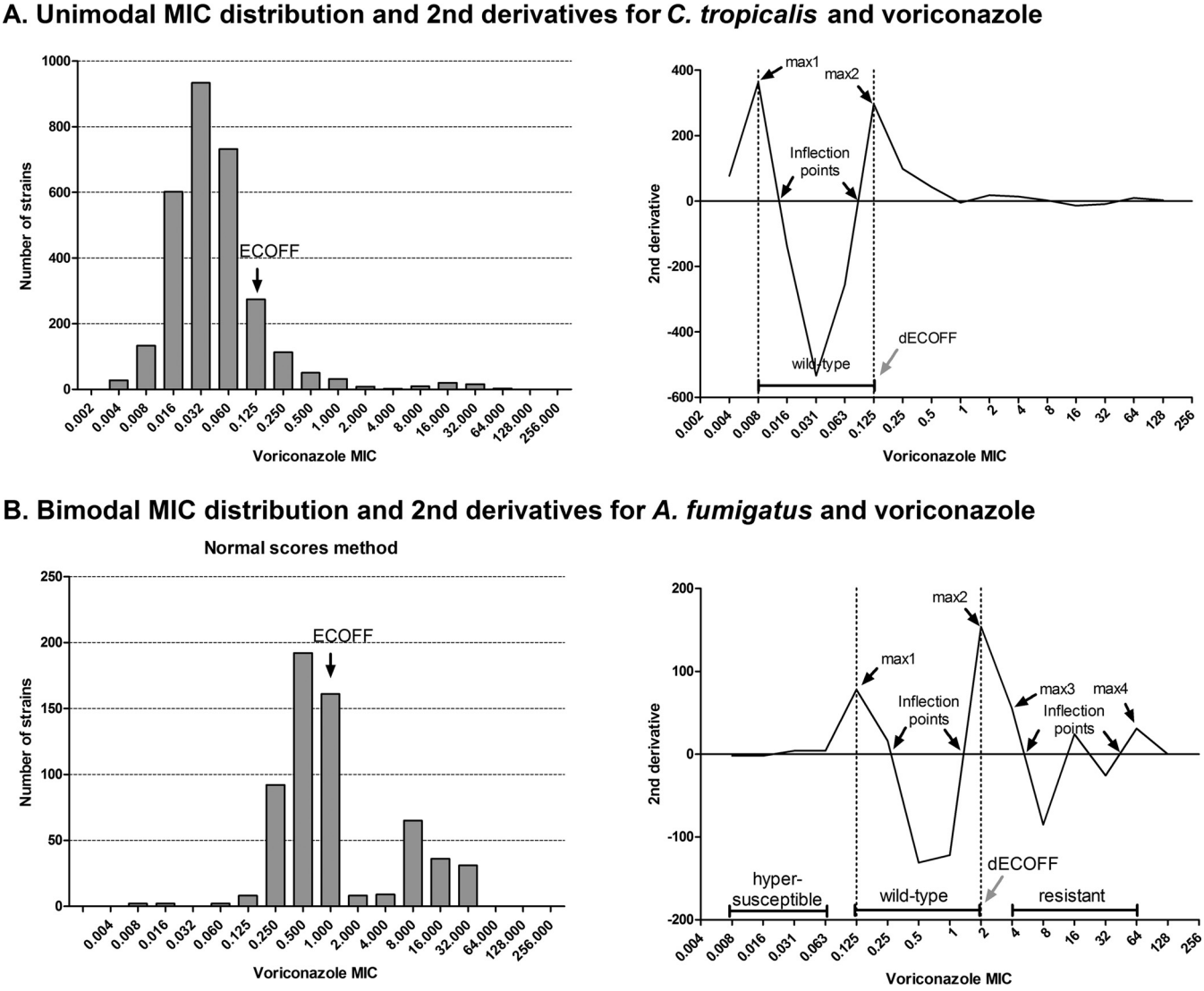

FIG 1 Examples of the derivatization method used with two previously published distributions of voriconazole. (Left) MIC distributions. ECOFFs were determined using the eyeball method. (Right) Second derivatives as functions of the MIC. ECOFFs were determined using the derivatization method. Inflection points, maximum second derivatives, and hypothetical MIC subpopulations are indicated.

whereas MIC subpopulations can also be determined between other maxima of second derivatives (Fig. 1). The absolute (0 2-fold-dilution difference) and essential (1 2-fold-dilution difference) agreements between EUCAST ECOFFs determined with the eyeball method (eyeball ECOFFs) and dECOFFs were then calculated for all drug-species combinations.

Based on the EUCAST breakpoints and ECOFFs (Table 1), the highest non-wild-type and resistance rates were found for *C. parapsilosis* against fluconazole (9.8%), voriconazole (5.8%), posaconazole (2.7%), and itraconazole (0.8%) (Table 2). The corresponding rate of *C. albicans* resistance to voriconazole was 7%, and it was 1% for the other azoles. Similarly, azole resistance rates for *C. tropicalis* were low (0.6 to 1.2%). For *C. glabrata*, 1.2 to 2.3% were resistant to all azoles, whereas for *C. krusei*, no isolates resistant to any azoles except fluconazole were found. For echinocandins, 3.3% of *C. krusei* and 1.1% of *C. tropicalis* isolates were resistant to anidulafungin, and 0.7% of *C. albicans* and 1.7% of *C. tropicalis* isolates were resistant to micafungin. Amphotericin B resistance was found only in *C. tropicalis* isolates (1.1%).

The principle of dECOFF determination is shown in Fig. 1. The ECOFFs determined by the eyeball and derivatization methods are presented in Table 1 for all drug-species

TABLE 2 MIC distribution for each drug and *Candida* species in this study

Species	Drug	No. of isolates showing resistance to indicated drug at an MIC (mg/liter) of:															MIC_{50}	MIC_{90}	R^b
		>32	32	16	8	4	2	1	0.5	0.25	0.125	0.063	0.031	0.016	0.008	0.004			
C. albicans (n = 584)	Amphotericin B							29	346	199	1						0.5	0.5	0.0
	Fluconazole	4		3	1	1	3	3	54	386	126	3					0.25	0.5	1.4
	Itraconazole								1	2	2	6	49	524			≤0.016	0.0315	0.9
	Voriconazole						1	2	2		1	4	4	570			≤0.016	≤0.016	7.0
	Posaconazole									1	5	5	139	434			≤0.016	0.0315	1.0
	Anidulafungin												3	133	448		≤0.008	0.016	0.0
	Micafungin											3	1	243	169	162	0.008	0.016	0.7
C. tropicalis (n = 180)	Amphotericin B						1	72	11	6							0.5	1	1.1
	Fluconazole				1	1	3	7	1	67	1						0.5	0.5	1.2
	Itraconazole									1	1	21	98	59			0.031	0.062	0.6
	Voriconazole									1	3	14	79	83			0.031	0.031	0.6
	Posaconazole										2	32	138	8			0.031	0.063	1.1
	Anidulafungin							1			1	21	77	78	2		0.031	0.063	1.1
	Micafungin										3	21	89	67			0.031	0.063	1.7
C. parapsilosis (n = 122)[a]	Amphotericin B							86	36								1	1	0.0
	Fluconazole		2	8	2	3	7	44	48	8							1	4	9.8
	Itraconazole									1	7	46	56	12			0.031	0.063	0.8
	Voriconazole								2	1	3	8	33	66			≤0.016	0.125	2.7
	Posaconazole										5	76	4	1			0.063	0.063	5.8
	Anidulafungin					3	38	81									1	2	0.0
	Micafungin					1	55	66									1	2	0.0
C. glabrata (n = 86)	Amphotericin B							27	46	13							0.5	1	0.0
	Fluconazole	2	1	3	5	39	31	5									4	8	2.3
	Itraconazole			1				1	8	37	25	11	3				0.25	0.5	1.2
	Voriconazole				1			1	2	8	35	3	9				0.125	0.25	1.7
	Posaconazole			1			1		2	39	37	6					0.125	0.25	2.3
	Anidulafungin											11	5	24	1		0.031	0.063	0.0
	Micafungin												9	59	12	6	0.016	0.0315	0.0
C. krusei (n = 30)	Amphotericin B							23	7								1	1	0.0
	Fluconazole	9	17	4													32	>64	100
	Itraconazole								3	12	11	3	1				0.125	0.25	0.0
	Voriconazole							4	8	16	2						0.25	1	0.0
	Posaconazole									6	16	7	1				0.125	0.25	0.0
	Anidulafungin										1	26	3				0.063	0.063	3.3
	Micafungin									8	21	1					0.125	0.25	0.0
Candida spp. (n = 97)	Amphotericin B							20	53	18	4	2					0.5	1	ND
	Fluconazole		1	1	7	20	15	9	22	19	3						1	4	ND
	Itraconazole								7	14	15	14	34	13			0.063	0.25	ND
	Voriconazole									7	12	16	12	50			≤0.016	0.125	ND
	Posaconazole								1	11	26	25	19	15			0.063	0.25	ND
	Anidulafungin			1		1	9	20	17	2	2	15	12	6	12		0.25	2	ND
	Micafungin				1			20	17	12	16	8	14	9			0.25	1	ND

[a]The MIC_{90}s of fluconazole, voriconazole, and echinocandins were 1 2-fold dilution higher for *C. parapsilosis sensu stricto* than for sibling species.
[b]Percent of resistant or non-wild-type isolates.

pairs. Of 35 comparisons (5 species, 7 drugs), the dECOFF was the same in 16 (46%), 1 dilution different in 17 (49%) (8 were higher, 9 were lower), and 2 dilutions lower in 2 (5%) (voriconazole with *C. albicans* and *C. parapsilosis*) compared to EUCAST ECOFFs. For *C. albicans* and *C. parapsilosis* and for all azoles, the dECOFFs were lower than the EUCAST ECOFFs. In the present study, we developed a simple objective nonparametric approach for ECOFF determination which combines the flexibility of the EUCAST approach and the robustness of other statistical approaches. The method is based on derivatization of an MIC frequency distribution and calculation of second derivatives. This describes mathematically the change in curvature of the MIC distribution. The ECOFF is then determined as the second maximum of the second derivatives of the MIC distribution, which corresponds to the MIC with the largest convexity at the right tail of the unimodal distribution. This approach can be applied to any shape of unimodal or multimodal MIC distribution, even in truncated data sets, since the exact shape (i.e., precise parameter estimates) of the Gaussian distribution is not needed. For multimodal

distributions, more than two maxima of the second derivatives are present, corresponding to hypersusceptible or resistant subpopulations (Fig. 1). A cutoff for the percentage of isolates that should be encompassed in the wild-type distribution is not needed, thus avoiding a major issue of controversy in the CLSI approach (3). It is also expected to be less sensitive for individual MIC distributions independent of median/modal MICs, SDs, and MIC frequencies, as long as the overall curvature is not changed.

Comparable ECOFFs, with some notable exceptions, were found with the eyeball method. Despite similar MIC distributions for micafungin and anidulafungin for *C. parapsilosis*, different eyeball ECOFFs were determined (2 versus 4 mg/liter, respectively), whereas dECOFFs were the same (4 mg/liter). Similarly, for *C. albicans*, current EUCAST ECOFFs of micafungin and anidulafungin correspond to the modal MICs (0.016 and 0.031 mg/liter, respectively), whereas the dECOFFs lie 1 dilution higher. Amphotericin B dECOFFs were consistently 1 dilution higher than the eyeball ECOFFs, probably because of heavy clustering of MICs within 2 to 4 dilutions, resulting in an MIC distribution with no tails. On the contrary, voriconazole and itraconazole dECOFFs were usually 1 dilution lower than eyeball ECOFFs because of long-tailed MIC distributions. These two types of MIC distributions with low and high kurtosis pose a real challenge to the eyeball method, since the real ECOFF is between 2 dilutions (in case of amphotericin B) and several dilutions (in case of azoles). The derivatization method may provide a statistical objective alternative tool to the eyeball method for ECOFF determination, particularly for those MIC distributions. It would be interesting to perform this method with larger and different MIC distributions for both bacterial and fungal species in a multicenter study that includes isolates with known resistance mechanisms.

In the present collection, resistance/non-wild-type rates were low for amphotericin B (<1%) and echinocandins (<3%), whereas for azoles, the highest rates were found for *C. parapsilosis* with fluconazole (10%) and voriconazole (6%) and for *C. albicans* with voriconazole (7%). These rates are comparable with those from previously published epidemiological studies, but with notable differences (8–12). Higher rates of resistance for *C. tropicalis* to voriconazole (25 to 67%) and of different *Candida* species to posaconazole (18 to 80%) were previously reported using smaller collections of bloodstream isolates (n <60) and a lower breakpoint of 0.06 mg/liter for posaconazole compared with that in the present study (8, 9, 11). Ten to 20% rates of resistance to echinocandins were reported for *C. albicans* (n = 97), *C. parapsilosis* (n = 55), and *C. glabrata* (n = 32) (9). These discrepancies emphasize the impact of the origin, the number of isolates tested, the collection period, and the breakpoint used on reported resistance rates in each study. Therefore, the new approach described here may help to determine in a simple, objective, and mathematically based manner local and global ECOFFs for detecting non-wild-type isolates.

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We declare no conflicts of interest.

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