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Epidemiological Cutoff Values for Fluconazole, Itraconazole, Posaconazole, and Voriconazole for Six *Candida* Species as Determined by the Colorimetric Sensititre YeastOne Method

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In the absence of clinical breakpoints (CBP), epidemiological cutoff values (ECVs) are useful to separate wild-type (WT) isolates (without mechanisms of resistance) from non-WT isolates (those that can harbor some resistance mechanisms), which is the goal of susceptibility tests. Sensititre YeastOne (SYO) is a widely used method to determine susceptibility of *Candida* spp. to antifungal agents. The CLSI CBP have been established, but not for the SYO method. The ECVs for four azoles, obtained using MIC distributions determined by the SYO method, were calculated via five methods (three statistical methods and based on the MIC_{50} and modal MIC). Respectively, the median ECVs (in mg/liter) of the five methods for fluconazole, itraconazole, posaconazole, and voriconazole (in parentheses: the percentage of isolates inhibited by MICs equal to or less than the ECVs; the number of isolates tested) were as follows: 2 (94.4%; 944), 0.5 (96.7%; 942), 0.25 (97.6%; 673), and 0.06 (96.7%; 849) for *Candida albicans*; 4 (86.1%; 642), 0.5 (99.4%; 642), 0.12 (93.9%; 392), and 0.06 (86.9%; 559) for *C. parapsilosis*; 8 (94.9%; 175), 1 (93.7%; 175), 2 (93.6%; 125), and 0.25 (90.4%; 167) for *C. tropicalis*; 128 (98.6%; 212), 4 (95.8%; 212), 4 (96.0%; 173), and 2 (98.5; 205) for *C. glabrata*; 256 (100%; 53), 1 (98.1%; 53), 1 (100%; 33), and 1 (97.9%; 48) for *C. krusei*; 4 (89.2%; 93), 0.5 (100%; 93), 0.25 (100%; 33), and 0.06 (87.7%; 73) for *C. orthopsilosis*. All methods included ≥94% of isolates and yielded similar ECVs (within 1 dilution). These ECVs would be suitable for monitoring emergence of isolates with reduced susceptibility by using the SYO method.

An important cause of morbidity and mortality in hospitalized patients with serious underlying conditions is candidemia, which is related to high health care costs (1, 2). However, the number of antifungal agents for candidemia treatment is small (azoles, echinocandins, flucytosine, and polyenes), and treatment failures have been reported. It is anticipated that antifungal susceptibility tests will be performed, particularly when this failure occurs, and additional epidemiological studies need to be performed. The Sensititre YeastOne (SYO) method is one of the antifungal susceptibility tests most frequently used in clinical laboratories. Clinical breakpoints (CBP) recommended by the CLSI have to be applied when tests are performed using that methodology. However, CBP are not available for the SYO method; therefore, in their absence, epidemiological cutoff values (ECVs) are useful for separation of isolates without mechanisms of resistance (wild-type [WT] from non-wild-type isolates, those that may harbor some resistance mechanisms [non-WT]), which is the goal of susceptibility tests. Several methods have been described to calculate ECVs; for instance, Arendrup et al. (3) estimated the ECVs as 2-fold dilution steps higher than the MIC_{50} (the MIC that inhibits 50% of isolates); Rodriguez-Tudela et al. (4) worked with 2-fold dilutions above the modal MIC; Cantón et al. (5), Kronvall (6), and Turnidge et al. (7) calculated the ECVs by using statistical methods, and the latter two study groups concluded that the values thus obtained fit well with those determined through the MIC_{50} or modal MIC methods. We previously reported the ECVs of echinocandins, amphotericin B, and flucytosine based on this methodology for the SYO method (5), and as an extension we have now determined the ECVs for the azole agents used for treatment of invasive *Candida* infections as recommended in the current guidelines (of the Infectious Diseases Society of America, FDA, etc.) (8).

The aims of this study were to (i) define the WT MIC distributions of fluconazole (FZ), itraconazole (ITR), posaconazole (POS), and voriconazole (VOR) by the SYO method for four of the most common and two less common *Candida* species that cause bloodstream infections, (ii) propose the ECVs for each species-drug combination, and (iii) compare the ECVs obtained by the five methods with each other and also with those obtained using the CLSI methodology.

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TABLE 1 Resistance mechanisms and MICs of azole agents determined with SYO for azole-resistant *Candida* spp.

Species	Strain	Mechanism(s) of resistance	MIC (μg/ml)			
			FZ	ITR	POS	VOR
C. parapsilosis	$BC014R_{VRC}$	Mutation K873N in Mrr1	64	0.125	0.125	0.5
C. albicans	95-190	Overexpression CDR1, CDR2, ERG11, and MDR1	256	16	8	8
C. parapsilosis	$BC014R_{FCZ}$	Mutation G583R in Mrr1	64	0.125	0.06	0.125

MATERIALS AND METHODS

Isolates. A total of 2,119 *Candida* bloodstream isolates were tested: 857 isolates were isolated from just one center (334 *C. albicans*, 315 *C. parapsilosis*, 56 *C. glabrata*, 62 C. *tropicalis*, 63 *C. orthopsilosis*, and 27 *C. krusei*) from January 1995 to December 2010, and 1,262 isolates (610 *C. albicans*, 327 *C. parapsilosis*, 156 *C. glabrata*, 113 *C. tropicalis*, 30 *Candida orthopsilosis*, and 26 *C. krusei*) were obtained from 43 public tertiary care hospital laboratories, which represent all Spanish geographical areas (FUNGEMYCA Epidemiological Study). Isolates were recovered from January 2009 to February 2010. Each isolate represented one infectious episode per patient and was identified by standard methods in each center, stored in a water suspension, and sent to the reference center (Hospital Universitario La Fe, Valencia, Spain) for posterior studies. *C. parapsilosis* and *C. orthopsilosis* strains were identified by a molecular methodology described elsewhere (9). Three azole-resistant *Candida* strains with different mechanisms of resistance were used to assess the accuracy of the ECVs calculated (Table 1) (10, 11).

Antifungal susceptibility testing. Susceptibility tests were performed at the participating hospitals on the first isolate from each candidemia episode by using the microdilution colorimetric SYO method, with the SYO-09 panel (TREK Diagnostic Systems, Cleveland, OH), as instructed in the commercial guidelines. The quality control strains *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were first tested in each participating laboratory, and results were sent to the reference center. All MIC values were within the expected ranges.

Definitions. The definitions of the WT population and ECV were those reported previously by other authors (4, 12, 13); briefly, a WT organism was defined as a strain that did not harbor any acquired or mutational resistance mechanism to the particular antifungal agent being examined.

The ECV is the highest MIC value for the WT population. It is calculated by taking into account the MIC distribution, the modal MIC of each distribution, and the inherent variability of the test (usually within one doubling dilution), and it should encompass ≥95% of isolates. The number of isolates needed to calculate a representative ECV was not established, but there is a consensus among experts that recommends at least 50 strains from at least three to five different laboratories be included.

Statistical analysis. Data were analyzed with R software (version 2.14.2) (14). Both on-scale and off-scale MICs were included, with the values left unchanged. In order to approach a normal distribution, the MICs were converted to $\log_2$ values. Statistical ECVs were calculated following the methods described by Turnidge et al., by Kronvall, and by the clustering method using the Mclust Library for R (version 3.4.11), as previously described (5–7). Since the ECVs estimated fell within a continuous scale, values were rounded to the nearest highest dilution after reconversion to concentration units.

RESULTS AND DISCUSSION

The MIC data for each species, including those species with few isolates (less than 100), and each antifungal agent were obtained from more than five laboratories. The MIC results of the antifungal agents tested, which have been reported elsewhere (15), were

TABLE 2 Wild-type MIC distributions of azole agents for six species of *Candida* based on the Sensititre YeastOne method

Agent	Species	No. of isolates with MIC (μg/ml) of:															Total no. of isolates
		0.008	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	
Fluconazole	*C. albicans*				9	98	302	340	118	24	13	10	2	2	1	6	19	944
	C. parapsilosis				1	14	37	137	190	103	71	55	18	12	4			642
	C. tropicalis					2	10	32	68	38	10	6				1	8	175
	C. glabrata					3		4	17	25	37	44	51	15	6	7	3	212
	C. krusei										1	3	4	7	20	18		53
	C. orthopsilosis					1	3	17	39	15	8	5	3		2			93
Itraconazole	*C. albicans*	36	131	204	275	225	35	5	2		1	4	24					942
	C. parapsilosis	8	40	97	186	218	79	10	4									642
	C. tropicalis		2	7	15	64	54	16	6	4		2	5					175
	C. glabrata		4	3	3	26	51	65	38	9	4	2	7					212
	C. krusei		1	2		6	25	13	5		1							53
	C. orthopsilosis	1	2	14	30	31	15											93
Posaconazole	*C. albicans*	69	177	210	150	47	4	4		1	2	9						673
	C. parapsilosis	21	80	124	91	52	19	1	3	1								392
	C. tropicalis	4	5	8	16	27	42	12	3		1	7						125
	C. glabrata	2	3		7	10	23	45	59	15	2	7						173
	C. krusei		1	1		2	20	6	3									33
	C. orthopsilosis		4	9	9	10	1											33
Voriconazole	*C. albicans*	688	95	27	11	8	5	2			3	8	2					849
	C. parapsilosis	185	143	92	66	45	15	7	5	1								559
	C. tropicalis	3	19	44	37	35	13	6		1	1	5	3					167
	C. glabrata	10	8	13	39	39	62	16	10	5	2	1						205
	C. krusei			1	1	16	21	6	2	1								48
	C. orthopsilosis	27	16	11	10	5	3	1										73

TABLE 3 Comparison of ECVs obtained for the four azole agents when we used the different methods

Species	No. of isolates tested	Agent	ECV obtained by indicated method (%)[a]						
			MIC_{50} + 2 dilutions	Modal MIC + 2 dilutions	Turnidge et al.	Kronvall	Clustering	Median of all studied methods	CLSI[b]
C. albicans	944	FZ	2 (94.4)	2 (94.4)	2 (94.4)	8 (96.8)	2 (94.4)	2 (94.4)	0.5 (98.1)
	942	ITR	0.25 (96.2)	0.25 (96.2)	0.5 (96.7)	1 (96.9)	2 (96.9)	0.5 (96.7)	0.12 (95.0)
	673	POS	0.12 (97.0)	0.12 (97.0)	0.25 (97.6)	0.5 (98.2)	0.5 (98.2)	0.25 (97.6)	0.06 (98.4)
	849	VOR	0.03 (95.4)	0.06 (96.7)	0.12 (97.6)	0.12 (97.6)	0.06 (96.7)	0.06 (96.7)	0.03 (99.0)
C. parapsilosis	642	FZ	4 (86.1)	4 (86.1)	8 (94.7)	16 (97.5)	4 (86.1)	4 (86.1)	2 (93.2)[c]
	642	ITR	0.25 (97.8)	0.5 (99.4)	0.5 (99.4)	0.5 (99.4)	0.25 (97.8)	0.5 (99.4)	0.5 (99.7)
	392	POS	0.12 (93.9)	0.12 (93.9)	0.25 (98.7)	0.5 (99.0)	0.12 (93.9)	0.12 (93.9)	0.25 (99.3)[c]
	559	VOR	0.06 (86.9)	0.06 (86.9)	0.06 (86.9)	0.25 (97.7)	0.03 (75.1)	0.06 (86.9)	0.12 (97.8)[c]
C. tropicalis	175	FZ	4 (91.4)	4 (91.4)	8 (94.9)	32 (94.9)	64 (94.9)	8 (94.9)	2 (98.4)
	175	ITR	0.5 (90.3)	0.5 (90.3)	1 (93.7)	4 (96)	2 (96)	1 (93.7)	0.5 (97.8)
	125	POS	1 (93.6)	1 (93.6)	2 (93.6)	4 (94.4)	4 (94.4)	2 (93.6)	0.12 (97.8)
	167	VOR	0.25 (90.4)	0.12 (82.6)	0.25 (90.4)	2 (94.6)	1 (94.0)	0.25 (90.4)	0.06 (97.3)
C. glabrata	212	FZ	32 (92.5)	64 (95.3)	128 (98.6)	128 (98.6)	128 (98.56)	128 (98.6)	32 (91.5)
	212	ITR	2 (93.9)	2 (93.9)	4 (95.8)	8 (96.7)	4 (95.8)	4 (95.8)	2 (95.2)
	173	POS	2 (94.8)	4 (96.0)	8 (100)	8 (100)	2 (94.8)	4 (96.0)	2 (96.1)
	205	VOR	0.5 (91.2)	1 (96.1)	2 (98.5)	2 (98.5)	2 (98.5)	2 (98.5)	0.5 (90.4)
C. krusei	53	FZ	256 (100)	256 (100)	128 (100)	256 (100)	128 (100)	256 (100)	64 (99.8)
	53	ITR	1 (98.1)	1 (98.1)	2 (98.1)	2 (98.1)	1 (98.1)	1 (98.1)	1 (99.0)
	33	POS	1 (100)	1 (100)	2 (100)	2 (100)	1 (100)	1 (100)	0.5 (99)
	48	VOR	1 (97.9)	1 (97.9)	1 (97.9)	1 (97.9)	1 (97.9)	1 (97.9)	0.5 (99.4)
C. orthopsilosis	93	FZ	4 (89.2)	4 (89.2)	8 (94.6)	32 (97.9)	2 (80.7)	4 (89.2)	2 (98)
	93	ITR	0.25 (100)	0.5 (100)	0.5 (100)	0.5 (100)	0.5 (100)	0.5 (100)	ND[d]
	33	POS	0.25 (100)	0.5 (100)	0.5 (100)	0.25 (100)	0.12 (97.0)	0.25 (100)	0.25 (97.1)
	73	VOR	0.06 (87.7)	0.03 (74.0)	0.06 (87.7)	0.5 (100)	0.06 (87.7)	0.06 (87.7)	0.06 (98)

[a] Percentage of isolates for which the MIC was less than or equal to the ECV (in μg/ml).
[b] Data were obtained from references 19 to 22.
[c] *C. parapsilosis* complex.
[d] ND, not determined.

similar to those observed by other authors who have used the SYO method (16–18). Table 2 describes the WT MIC distributions of the four azoles tested. All MIC distributions were typical for WT organisms and covered three to five 2-fold dilution steps surrounding the modal MIC, except those of ITR ($n = 93$) and POS ($n = 33$) for *C. orthopsilosis*, for which only 1 dilution was above the modal MIC. Table 3 depicts the ECVs obtained with the SYO method, as determined with the five methods mentioned above (three statistical and two using dilutions above the MIC_{50} or the modal MIC), the median ECVs of the five methods, and those published by Pfaller et al. for the CLSI. Although the fluconazole MIC for *C. krusei* is not recommended, the ECV was included in this table because this drug is incorporated in the SYO 09 panel and for comparison with the CLSI ECVs. The same was true for the echinocandin ECVs; the statistical method proposed by Turnidge et al. gave lower ECVs than those proposed by Kronvall, although 70.8% were within 1 dilution. There was only a difference of three 2-fold dilutions for the ECVs of VOR for *C. tropicalis* (0.25 versus 2 mg/liter) and *C. orthopsilosis* (0.06 versus 0.5 mg/liter). By the clustering method, 75% of ECVs were within one 2-fold dilution of those obtained with Turnidge et al.'s method; the greatest differences were three 2-fold dilutions for the ECVs of FZ for *C. tropicalis* (8 versus 64 mg/liter), and the rest of the ECVs were within the 2-fold dilutions. The median ECVs were, in general, equal to or two 2-fold dilutions higher than those of the CLSI value, with the exception of POS for *C. tropicalis*, for which the ECV was four 2-fold dilutions higher (2 versus 0.12 mg/liter). The ECVs obtained with the modal MIC and MIC_{50} were in general lower than the values obtained when we applied the statistical methods; nevertheless, 100 and 91.7% of the cases, respectively, were within 1 dilution of the median ECV (Tables 3 and 4). The MIC values determined with the SYO method for *Candida* strains with different mutations used to confirm the validity of the ECVs are shown in Table 1; all methods used to calculate the ECVs classified the strains harboring mutations as non-WT.

The ECVs obtained by the different methods were compared with each other and with those reported based on the CLSI methodology. Therefore, the median values for the five methods analyzed were those put forward as the tentative ECVs. Table 4 shows the level of agreement (within one and two 2-fold dilutions) between methods, as well as the cases where the difference was greater than two 2-fold dilutions, and it also includes the Pearson's correlation values. In general, the agreement was ≥95.7%. The most repetitive exception was the ECV of POS for *C. tropica-*

TABLE 4 Pearson's correlation and comparison of the ECVs for the SYO method obtained with different techniques

Methods compared	R	% agreement for ECVs at indicated dilution		
		±1 dilution	±2 dilutions	>2 dilutions
CLSI vs median	0.99	60.9	95.7	POS for *C. tropicalis*
CLSI vs clustering	0.89	56.5	78.3	ITR and POS for *C. albicans*; FZ, VOR, and POS for *C. tropicalis*
CLSI vs Turnidge et al.	0.94	43.5	95.7	POS for *C. tropicalis*
CLSI vs Kronvall	0.99	23.1	56.5	FZ for *C. albicans*, *C. parapsilosis*, *C. tropicalis*, and *C. orthopsilosis*; ITR and POS for *C. albicans* and *C. tropicalis*; VOR for *C. tropicalis* and *C. orthopsilosis*
CLSI vs mode	0.97	86.95	95.7	POS for *C. tropicalis*
CLSI vs MIC_{50}	0.94	82.7	95.7	POS for *C. tropicalis*
Median vs clustering	0.89	87.5	95.8	FZ for *C. tropicalis*
Median vs Turnidge et al.	0.94	100		
Median vs Kronvall	0.99	62.5	87.5	VOR for *C. tropicalis* and *C. orthopsilosis*; FZ for *C. orthopsilosis*
Median vs mode	0.97	100		
Median vs MIC_{50}	0.94	91.7	100	
Clustering vs Turnidge et al.	0.95	75	95.8	FZ for *C. tropicalis*
Clustering vs Kronvall	0.91	70.8	87.5	VOR for *C. parapsilosis* and *C. orthopsilosis*
Clustering vs mode	0.8	70.8	87.5	FZ and VOR for *C. tropicalis*
Clustering vs MIC_{50}	0.73	66.6	91.6	FZ for *C. tropicalis*; ITR for *C. albicans*
Turnidge vs Kronvall	0.94	70.8	91.6	VOR for *C. tropicalis* and *C. orthopsilosis*
Turnidge vs mode	0.84	100		
Turnidge vs MIC_{50}	0.77	83.3	100	
Kronvall vs mode	0.97	45.8	79.2	FZ, ITR, and VOR for *C. tropicalis*; VOR for *C. orthopsilosis*
Kronvall vs MIC_{50}	0.93	29.2	79.2	FZ, ITR, and VOR for *C. tropicalis*; FZ and VOR for *C. orthopsilosis*
Mode vs MIC_{50}	0.99	100		

lis, for which the CLSI ECV was 3 to 5 dilutions lower than those obtained with the other methods.

The Pearson's correlation between the median ECV from the SYO method and each of the other methods was very good (≥0.94), except for that from the clustering method (0.89) (Table 4). In a comparison of the ECVs from the CLSI method with those obtained with the SYO method for each antifungal agent, FZ showed the best correlation (>0.96), and when we compared species, the lowest correlation was for *C. tropicalis*. This species also showed the lowest Pearson's correlation for the echinocandin ECVs with the SYO method (5).

The ECVs were also calculated for the distribution of the two sources of isolates individually, i.e., 1,262 isolates from 43 laboratories and 857 isolates from 1 center. The same result occurred with the echinocandin ECVs, and the results obtained were, in general, the same as those found with all 2,119 isolates, or they fell within one 2-fold dilution, which is in agreement with the work of Turnidge et al. and Kronvall, who reported that their methods are also valid for data sets generated by a single laboratory and method (6, 7). Just as we emphasized in the determination of echinocandin ECVs with the SYO method, it is difficult to establish which statistical method best defines the ECV, due to the scarcity of resistant isolates. Perhaps the optimal method depends on the heterogeneity of the agent's MIC distributions, since different species may have different numbers of subpopulations and, consequently, different MIC distribution shapes (unimodal, bimodal, or multimodal). In fact, the majority of differences, in general, were only within one 2-fold dilution, and furthermore, the Pearson's correlation values between methods were good, and so we propose that the median values of the five methods used in this evaluation be considered the tentative ECVs for the SYO method. Moreover, independently of the method used, a 5% risk of misidentification was always assumed (in fact, it is 2.5%, because the distribution has two tails). In general, all ECVs determined with the five methods included ≥94% of isolates; the exceptions were the ECVs of FZ and VOR for *C. parapsilosis* and *C. orthopsilosis*. Other authors have also reported ECVs that did not include 95% of isolates (19, 20).

The tentative ECVs proposed are very similar to those of the CLSI; the differences in some values are the same as those found when comparing MIC values obtained with the CLSI or SYO methodology (16–18). The advantage of this study is that all isolates used were obtained from patients without prior treatment with any of the antifungal agents tested; thus, the outlier isolates probably represented mutant strains. The data came from 43 laboratories, and all isolates were obtained from blood cultures. Among the limitations of the study are the small numbers of *C. krusei* and *C. orthopsilosis* isolates tested, but we have kept the tentative ECVs for these species, for comparison among methods. Moreover, all isolates came from just one country, and so these values may not be completely representative but could be shared with other data sets in the future to establish forthcoming ECVs with the SYO method. The low number of strains with known mechanisms of resistance was another limitation. Nevertheless, these data have corroborated the ECVs obtained. We propose, for the first time, tentative ECVs for the SYO method and azole agents to help in monitoring the emergence of isolates with decreased susceptibilities to these agents, which is the aim of susceptibility tests and also of generation of ECVs.

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