AAC Journals.ASM.org

Multilaboratory Study of Epidemiological Cutoff Values for Detection of Resistance in Eight *Candida* Species to Fluconazole, Posaconazole, and Voriconazole

A. Espinel-Ingroff,[a] M. A. Pfaller,[b,c] B. Bustamante,[d] E. Canton,[e] A. Fothergill,[f] J. Fuller,[g] G. M. Gonzalez,[h] C. Lass-Flörl,[i] S. R. Lockhart,[j] E. Martin-Mazuelos,[k] J. F. Meis,[l,m] M. S. C. Melhem,[n] L. Ostrosky-Zeichner,[o] T. Pelaez,[p] M. W. Szeszs,[q] G. St-Germain,[r] L. X. Bonfietti,[s] J. Guarro,[t] J. Turnidge[u]

VCU Medical Center, Richmond, Virginia, USA[a]; JMI Laboratories, North Liberty, Iowa, USA[b]; University of Iowa College of Medicine, Iowa City, Iowa, USA[c]; Instituto de Medicina Tropical Alexander Von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru[d]; Unidad de Microbiologia Experimental, Centro de Investigacion, Hospital Universitario La Fe, Valencia, Spain[e]; University of Texas Health Science Center, San Antonio, Texas, USA[f]; The University of Alberta, Edmonton, Alberta, Canada[g]; Universidad Autonóma de Nuevo León, Monterrey, Nuevo León, México[h]; The Innsbruck Medical University, Division of Hygiene and Medical Microbiology, Innsbruck, Austria[i]; Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, USA[j]; Hospital Universitario de Valme, Seville, Spain[k]; Canisius-Wilhelmina Hospital, Nijmegen, Netherlands[l]; Radboud University, Nijmegen, Netherlands[m]; Fungal Taxonomy Laboratories, Adolfo Lutz Institute, São Paulo City, Brazil[n]; University of Texas Health Science Center, Houston, Texas, USA[o]; Hospital General Universitario Gregorio Marañón, Facultad de Medicina, Universidad Complutense, Madrid, Spain[p]; Mycology Department, Adolfo Lutz Institute, São Paulo City, Brazil[q]; Laboratoire de Santé Publique du Québec, Institut National de Santé Publique du Québec, Quebec, Canada[r]; Adolfo Lutz Institute, Araçatuba City, Brazil[s]; Facultat de Medicina, IISPV, URV, Reus, Spain[t]; University of Adelaide, Adelaide, Australia[u]

Although epidemiological cutoff values (ECVs) have been established for *Candida* spp. and the triazoles, they are based on MIC data from a single laboratory. We have established ECVs for eight *Candida* species and fluconazole, posaconazole, and voriconazole based on wild-type (WT) MIC distributions for isolates of *C. albicans* ($n = 11{,}241$ isolates), *C. glabrata* (7,538), *C. parapsilosis* (6,023), *C. tropicalis* (3,748), *C. krusei* (1,073), *C. lusitaniae* (574), *C. guilliermondii* (373), and *C. dubliniensis* (162). The 24-h CLSI broth microdilution MICs were collated from multiple laboratories (in Canada, Brazil, Europe, Mexico, Peru, and the United States). The ECVs for distributions originating from ≥6 laboratories, which included ≥95% of the modeled WT population, for fluconazole, posaconazole, and voriconazole were, respectively, 0.5, 0.06 and 0.03 μg/ml for *C. albicans*, 0.5, 0.25, and 0.03 μg/ml for *C. dubliniensis*, 8, 1, and 0.25 μg/ml for *C. glabrata*, 8, 0.5, and 0.12 μg/ml for *C. guilliermondii*, 32, 0.5, and 0.25 μg/ml for *C. krusei*, 1, 0.06, and 0.06 μg/ml for *C. lusitaniae*, 1, 0.25, and 0.03 μg/ml for *C. parapsilosis*, and 1, 0.12, and 0.06 μg/ml for *C. tropicalis*. The low number of MICs (<100) for other less prevalent species (*C. famata*, *C. kefyr*, *C. orthopsilosis*, *C. rugosa*) precluded ECV definition, but their MIC distributions are documented. Evaluation of our ECVs for some species/agent combinations using published individual MICs for 136 isolates (harboring mutations in or upregulation of *ERG11*, *MDR1*, *CDR1*, or *CDR2*) and 64 WT isolates indicated that our ECVs may be useful in distinguishing WT from non-WT isolates.

Severe candidal infections are seen worldwide among immunocompromised hosts and nonimmunocompromised patients. Irrespective of the species, these infections are associated with high mortality and morbidity rates (1, 2). In addition to the different amphotericin B formulations, the triazoles are recommended as primary (fluconazole and voriconazole) and prophylactic (fluconazole and posaconazole) treatments for invasive infections caused by *Candida* spp. (3, 4). The azoles block the pathway of ergosterol biosynthesis by inhibiting the 14-α-lanosterol demethylase enzyme. The wide use of fluconazole and other triazoles has led to *in vitro* resistance among *Candida* and other fungal isolates to fluconazole and, to a lesser extent, the newer triazoles, voriconazole and posaconazole (5). Various molecular mechanisms are associated with *in vitro* resistance to triazoles among *Candida* spp., such as (i) modifications in the quality or quantity of the target enzyme, reduced access of the drug to the target, mutations in the *ERG* genes participating in ergosterol biosynthesis, or a combination of these mechanisms, and (ii) active efflux of azole out of the cell through the activation of multidrug efflux transporters encoded by the *MDR* and *CDR* genes (6–12). The Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antifungal Susceptibility Tests has adjusted the breakpoints (BPs) for fluconazole and voriconazole to be species specific (13). A recent study defined triazole epidemiological cutoff values (ECVs) (12) based on data from a single laboratory for the triazoles and several species of *Candida*; however, BPs are not available for posaconazole and any fungal species or for the less prevalent species and fluconazole and voriconazole. The ECV, defined as the highest susceptibility endpoint of the wild-type (WT) population MIC, has been shown to detect the emergence of *in vitro* resistance or to separate WT isolates (without known mechanisms of resistance) from non-WT isolates (with mechanisms of resistance and reduced susceptibilities to the agent being evaluated) (12, 14–16). The data from multiple laboratories used to define ECVs in the present study should be more representative of the susceptibilities of these species to the triazoles evaluated.

The purpose of this study was (i) to define the wild-type susceptibility endpoint distributions of fluconazole, posaconazole, and voriconazole for 5 common and 3 less common *Candida* spp.

Received 1 December 2013 **Returned for modification** 1 January 2014
Accepted 5 January 2014
Published ahead of print 13 January 2014
Address correspondence to A. Espinel-Ingroff, avingrof@vcu.edu.

doi:10.1128/AAC.02615-13

TABLE 1 Pooled MIC distributions of fluconazole for 12 *Candida* species

Species	No. of labs[a]	No. of isolates	No. of isolates for which the MIC (μg/ml) was[b]:											
			0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	≥128
C. albicans	9[c]	5,265	254	1,729	1,647	855	370	137	91	59	48	37	26	12
C. dubliniensis	7[c]	162	18	54	55	21	5	1		2	3		2	1
C. glabrata	14	7,538		29	78	189	474	2,065	2,676	773	343	322	441	148
C. guilliermondii	11[c]	373		4	7	20	68	160	87	12	6	4	3	2
C. krusei	11	1,073			1	1	2	9	23	165	554	227	83	8
C. lusitaniae	10[c]	574	1	76	181	199	64	12	4	8	8	15	6	
C. parapsilosis	15	6,023	8	233	2,021	2,479	655	288	136	82	74	23	21	3
C. tropicalis	14	3,748	24	558	1,464	963	446	146	61	35	26	8	14	3
C. famata	9	49		9	16	12	7	2	3					
C. kefyr	7	36		5	16	12	1	1		1				
C. orthopsilosis	4	68		3	14	29	13	6	2	1				
C. rugosa	9	76		1	3	14	24	23	5	4	2			

[a] Number of laboratories contributing data to each MIC distribution.
[b] MICs determined at 24 h as described in the CLSI M27-A3 reference method (17). The modal MIC (most frequent value) for each distribution is underlined.
[c] Data from the other 1 to 4 labs were not used due to abnormal MIC distributions (the mode and lowest concentration tested were the same).

originating from ≥6 laboratories and (ii) to propose ECVs for these 3 triazoles using the 24-h CLSI broth microdilution method (17). We aggregated MICs obtained in 15 independent laboratories (29 to 11,241, species and agent dependent). MIC distributions for other less prevalent *Candida* species (*C. famata*, *C. kefyr*, *C. orthopsilosis*, *C. rugosa*) also are documented. In addition, since our isolates have not been assessed for mechanisms of resistance, we evaluated our ECVs using available studies where MICs for individual isolates, determined using broth microdilution methods, and the presence or absence of mechanisms of resistance were reported for some of the species included in the present study (6–8, 10, 18–26).

MATERIALS AND METHODS

Isolates. Each isolate originated from a unique clinical specimen from 1 of 18 independent laboratories. In the present study, the MICs of the three triazoles used for ECV definition were obtained at the following medical centers: VCU Medical Center, Richmond, VA; Instituto de Medicina Tropical Alexander Von Humboldt, Universidad Peruana Cayetano Heredia, Lima, Peru; Unidad de Microbiologia Experimental, Hospital Universitario La Fe, Valencia, Spain; University of Texas Health Science Center, San Antonio, TX; The University of Alberta, Edmonton, Alberta, Canada; Universidad Nacional Autónoma de México, Mexico; The Innsbruck Medical University, Innsbruck, Austria; Centers for Disease Control and Prevention, Atlanta, GA; Hospital Universitario de Valme, Seville, Spain; Department of Medical Microbiology and Infectious Diseases, Canisius-Wilhelmina Hospital, Nijmegen, Netherlands; The Adolfo Lutz Institute, São Paulo City, Brazil; University of Texas Health Science Center, Houston, TX; Hospital General Universitario Gregorio Marañón, Faculty of Medicine, Universidad Complutense, Madrid, Spain; University of Iowa, Iowa City, IA; Mycology Department, Adolfo Lutz Institute, São Paulo City, Brazil; Institut National de Santé Publique du Québec, Laboratoire de Santé Publique du Québec, Quebec, Canada; Adolfo Lutz Institute, Araçatuba City, Brazil; Facultat de Medicina, IISPV, URV, Reus, Spain. These laboratories were coded 1 to 20 (for several studies), but because some laboratories were excluded from the study or did not provide triazole MIC data for some species, we used data from the remaining 15 laboratories. Species were identified and stored at each medical center using standard and molecular methodologies (27), and isolates were not evaluated for azole resistance mechanisms.

We aggregated the available 24-h CLSI MIC data of each agent for 11,241 *C. albicans*, 162 *C. dubliniensis*, 7,538 *C. glabrata*, 373 *C. guilliermondii*, 1,073 *C. krusei*, 574 *C. lusitaniae*, 6,023 *C. parapsilosis*, and 3,748 *C. tropicalis* isolates originating from 6 to 15 different laboratories and for four other less prevalent species (49 *C. famata*, 36 *C. kefyr*, 68 *C. orthopsilosis*, and 76 *C. rugosa* isolates) from 3 to 9 different laboratories (Tables 1 to 3). One or both quality control (QC) isolates (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) were used by the participating laboratories (13, 17).

In addition, we included triazole MIC distributions from previously published studies (5 species, 73 to 200 isolates, 20 to 64 WT MICs and 53 to 136 non-WT MICs [agent dependent]), all tested for the presence (non-WT) or absence (WT) of either intrinsic or acquired azole resistance mechanisms (e.g., substitutions and missense mutations in or upregulation of *ERG11*, *MDR1*, *CDR1*, and *CDR2*), in order to assess the ability of the various fluconazole, posaconazole, and voriconazole ECVs to discriminate non-WT from WT strains of *Candida* spp. at the molecular level (6–8, 10, 18–26).

Antifungal susceptibility testing. The MICs were obtained at each center by following the CLSI M27-A3 broth microdilution method (standard RPMI 1640 broth [0.2% dextrose], final inoculum concentrations that ranged from 0.5×10^3 to 5×10^3 CFU/ml, and 24 h of incubation); MICs were the lowest drug concentrations that produced ≥50% growth inhibition compared to the growth control (17). MIC data for the two QC reference strains, utilized during the years of testing in each center, were obtained each time that a set of isolates was tested following the CLSI M27-A3 broth microdilution method (13, 17). The majority of MIC ranges (98 to 100%) for the two QC strains were within the CLSI established reference range in each laboratory that had data included in the analyses; a certain degree of interlaboratory modal variability (mostly ±2-fold dilution) was observed.

Definitions. The ECV (also known as the wild-type cutoff, or CO_{WT}) definition and the definitions of the two populations (WT and non-WT MIC populations or isolates) that will be discussed have been provided above (12, 14–16, 28). Briefly, a non-WT organism shows reduced susceptibility to the agent being evaluated compared to the WT (without resistant mechanisms) population, but it may or may not respond to treatment with the drug being evaluated. ECVs are 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 should encompass ≥95% of isolates (28).

Data analysis. As previously described (28–32), the MIC distribution of each species obtained in each coded laboratory (numbered 1 to 20) was listed in an Excel spreadsheet and screened for (i) grossly skewed distributions that precluded statistical fitting (distributions that had a modal MIC [most frequent value] at the lowest or highest concentration tested and/or which were bimodal in the presumptive wild-type distribution),

TABLE 2 Pooled MIC distributions of posaconazole for 12 *Candida* species

Species	No. of labs[a]	No. of isolates	No. of isolates for which the MIC (μg/ml)was[b]:										
			0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	≥8
C. albicans	9[c]	11,241	976	5,197	3,365	1,230	259	132	55	13	3	1	10
C. dubliniensis	7	151	2	14	51	38	35	8	3				
C. glabrata	7	2,131	7	59	127	193	386	602	520	153	46	18	20
C. guilliermondii	6	298	1	9	28	38	115	79	22	4	2		
C. krusei	10	872		5	37	109	253	356	87	20	3	2	
C. lusitaniae	7[c]	521	8	199	167	97	37	8	2	3			
C. parapsilosis	7[c]	3,451	43	446	1,033	1,024	678	165	45	13	4		
C. tropicalis	8	2,613	103	661	860	646	249	50	20	6	6	4	8
C. famata	6	45		4	11	20	7	2	1				
C. kefyr	6	29			2	9	11	4	3				
C. orthopsilosis	3	55	1	8	24	15	7						
C. rugosa	8	45		4	11	20	7	2	1				

[a] Number of laboratories contributing data to each MIC distribution.
[b] MICs determined at 24 h as described in the CLSI M27-A3 reference method (17). The modal MIC (most frequent value) for each distribution is underlined.
[c] Data from the other 2 to 4 labs were not used due to abnormal MIC distributions (the mode and lowest concentration tested were the same).

(ii) distribution size (data from ≥3 laboratories and the total pooled distribution had ≥100 isolates), and (iii) unusual modal variation (modes that were >2-fold dilutions from the others). Skewed distributions were removed from each pooled distribution of each species/agent used for the analysis (28, 31). The resulting screened and pooled MIC distributions were used to calculate the ECVs by the statistical method where the modeled population is based on fitting a normal distribution at the lower end of the MIC range, calculating the mean and standard deviation of that normal distribution, and using those parameters to calculate the MIC that captures at least 95%, 97.5%, and 99% of the modeled WT population (28).

RESULTS AND DISCUSSION

The ultimate goal of susceptibility testing is to predict with some reliability the clinical outcome when an infected patient is treated with the specific agent evaluated. The endpoint that categorizes an MIC as susceptible or resistant is the BP (14–16). However, in the fungal world, there are many species and agent combinations for which BPs have not been proposed. The reason for that is the lack of sufficient data correlating clinical outcomes and *in vitro* results used to establish BPs. That is the case for posaconazole and *Candida* spp., the other triazoles for some of the less prevalent *Candida* species, and for *C. glabrata* versus voriconazole (13). Although the ECV is not a BP, ECVs serve as an early indication of emerging changes in the patterns of susceptibility of organisms to the agent being evaluated. Species-specific ECVs have been previously defined based on MIC data from a single laboratory (12), which may not completely represent the WT MIC population for each species of *Candida* and each of the three triazoles evaluated in the present study. Because of this, we used data from multiple laboratories to define ECVs for fluconazole, posaconazole, and voriconazole, for the five most prevalent *Candida* spp., and for *C. dubliniensis*, *C. guilliermondii*, and *C. lusitaniae* (Tables 1 to 4). In addition, MIC distributions for another four less prevalent species are provided (Tables 1 to 3).

Eighteen laboratories submitted MIC data for the present study. MICs from between 1 and 7 laboratories, depending on the species and antifungal agent, were not included in the final analysis due to truncated distributions (modal MIC at the lowest concentration tested). All of the MIC distributions were typical for WT organisms and covered 3 to 5 2-fold dilution steps surrounding the modal MIC. The remaining aggregated MIC distributions for the three triazoles that originated in 3 to 15 laboratories are

TABLE 3 Pooled MIC distributions of voriconazole for 12 *Candida* species

Species	No. of labs[a]	No. of isolates	No. of isolates for which the MIC (μg/ml) was[b]:										
			0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	≥8
C. albicans	9[c]	3,210	105	1,768	670	291	140	114	84	21	11	2	4
C. dubliniensis	7	152	52	86	7	3	2	1		1			
C. glabrata	11	4,176	13	208	556	1,476	983	304	166	162	172	112	24
C. guilliermondii	12	369	3	42	126	119	33	20	12	8	1	3	2
C. krusei	12	930	1	8	36	136	476	207	53	12	1		
C. lusitaniae	8[c]	142	23	61	51	6		1					
C. parapsilosis	8[c]	2,337	188	986	547	334	119	80	46	26	11		
C. tropicalis	8[c]	3,127	547	912	893	441	155	69	43	31	21	8	7
C. famata	9	53	6	10	23	10	3	1					
C. kefyr	7	34	4	17	11	1	1						
C. orthopsilosis	4	66	9	30	17	9		1					
C. rugosa	9	59	1	6	34	12	3	3					

[a] Number of laboratories contributing data to each MIC distribution.
[b] MICs determined at 24 h as described in the CLSI M27-A3 reference method (17). The modal MIC (most frequent value) for each distribution is underlined.
[c] Data from 1 to 7 labs were not used due to abnormal MIC distributions (the mode and lowest concentration tested were the same).

TABLE 4 Epidemiological cutoff values from 6 to 15 laboratories as determined by the CLSI M27-A3 broth microdilution method

Antifungal agent	Species	No. of isolates/no. of labs	MIC (range) (μg/ml)	Mode (μg/ml)[a]	ECV (μg/ml) at the indicated % of the modeled WT population[b]	
					95	97.5
Fluconazole	*C. albicans*	5,265/9	0.06 to ≥128	0.12	0.5	0.5
	C. dubliniensis	162/7	0.06 to 64	0.25	0.5	0.5
	C. glabrata	7,538/14	0.12 to ≥128	4	8	8
	C. guilliermondii	373/11	0.12 to 64	2	8	8
	C. krusei	1,073/11	0.25 to ≥128	16	32	32
	C. lusitaniae	574/10	0.12 to 64	0.5	1	1
	C. parapsilosis	6,023/15	0.06 to ≥128	0.5	1	1
	C. tropicalis	3,748/14	0.06 to ≥128	0.25	1	1
Posaconazole	*C. albicans*	11,241/9	0.008 to ≥8	0.016	0.06	0.06
	C. dubliniensis	151/7	0.008 to 0.5	0.03	0.25	0.25
	C. glabrata	2,131/7	0.008 to ≥8	0.25	1	2
	C. guilliermondii	298/6	0.008 to 2	0.12	0.5	0.5
	C. krusei	872/10	0.016 to 4	0.25	0.5	0.5
	C. lusitaniae	521/7	0.008 to 1	0.016	0.06	0.06
	C. parapsilosis	3,451/7	0.008 to 2	0.03	0.25	0.25
	C. tropicalis	2,613/8	0.008 to ≥8	0.03	0.12	0.12
Voriconazole	*C. albicans*	3,210/9	0.008 to ≥8	0.016	0.03	0.03
	C. dubliniensis	152/7	0.008 to 1	0.016	0.03	0.03
	C. glabrata	4,176/11	0.008 to ≥8	0.06	0.25	0.25
	C. guilliermondii	369/12	0.008 to ≥8	0.03	0.12	0.12
	C. krusei	930/12	0.008 to 2	0.12	0.25	0.5
	C. lusitaniae	142/8	0.008 to 0.25	0.016	0.06	0.06
	C. parapsilosis	2,337/8	0.008 to 2	0.016	0.03	0.03
	C. tropicalis	3,127/8	0.008 to ≥8	0.016	0.06	0.12

[a] Most frequent MIC.
[b] Calculated ECVs comprising ≥95% or ≥97.5% of the statistically modeled population using pooled MICs originating from 6 to 15 laboratories.

shown in Tables 1 to 3. Overall, the distributions were quite normal; similar-sized "bars" were observed with some of the agent and species combinations (e.g., at fluconazole MICs of 0.12 and 0.25 μg/ml for *C. albicans* and 0.12 and 0.25 μg/ml for *C. dubliniensis*), which indicated that the mode lies between those two concentrations. The fluconazole modal MICs ranged from 0.12 to 16 μg/ml; the lower value was for *C. albicans*, and the highest was for *C. krusei* (Tables 1 and 4). The lowest posaconazole modes were for *C. albicans* and *C. lusitaniae* (0.016 μg/ml), and the highest were for *C. glabrata* and *C. krusei* (0.25 μg/ml). Overall, the voriconazole modes were lower (0.016 to 0.03 μg/ml) than those of the other two azoles for most of the species, with the exception of *C. glabrata* and *C. krusei* (voriconazole modes, 0.06 and 0.12 μg/ml, respectively). Our MIC distributions are similar to those observed by other authors (33–37). Previously reported azole MICs for *C. dubliniensis* (fluconazole MIC_{90} range, 8 to 32 μg/ml) and *C. guilliermondii* (fluconazole MIC_{90} range, 16 to 32 μg/ml; voriconazole MIC_{90} range, 4 to 8 μg/ml) were higher than those observed in the present study (Tables 1 to 3).

The triazole ECVs based on the aggregated MIC distributions of 8 of the 12 *Candida* spp. evaluated are shown in Table 4. Although the MIC distributions are provided for four less prevalent species (*C. famata*, *C. kefyr*, *C. orthopsilosis*, and *C. rugosa*), their ECVs were not calculated because the current criterion for ECV definition requires that the total pooled distribution have ≥100 isolates from ≥3 laboratories. The ECVs were defined using ≥95%, ≥97.5%, and ≥99% of the modeled MIC populations; we focused on the more conservative values (lower ECVs encompassing ≥95% of the modeled population). This decision was corroborated by the genetic information discussed below for the four more prevalent species and *C. dubliniensis*, although the ECVs encompassing ≥97% of the population were similar to those at 95%. ECVs defined using CLSI MIC data from a single laboratory are the same or 1 to 2 dilutions higher than those in the present study (12); in contrast, ECVs using YeastOne MIC data are mostly higher (38). The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has cutoff values for *Candida* and the azoles, and it has established ECVs (ECOFFs) for fluconazole, posaconazole, and voriconazole, respectively, of 1, 0.06, and 0.125 μg/ml for *C. albicans*, of 32, 1, and 1 μg/ml for *C. glabrata*, of 128, 0.5, and 1 μg/ml for *C. krusei*, and of 2, 0.06, and 0.12 μg/ml for *C. parapsilosis* and *C. tropicalis* (see http://www.EUCAST.org). For the most part, with the exception of *C. glabrata* and fluconazole, these values are comparable to those reported herein.

The ability of the ECVs encompassing 95% of the statistically modeled population to differentiate strains of *Candida* spp. with intrinsic or acquired azole resistance mechanisms (e.g., substitutions and missense mutations in or upregulation of *ERG11*, *MDR1*, *CDR1*, or *CDR2*) may be seen in the data presented in Table 5. The isolates in the collection depicted in Table 5 were compiled from 13 previously published studies to represent WT and non-WT MIC results for fluconazole, posaconazole, and vori-

TABLE 5 Application of ECVs to MIC distributions of fluconazole, posaconazole, and voriconazole versus *Candida* species strains tested for the presence of azole resistance mutations by broth microdilution methods[a]

Species	Antifungal agent (no. of isolates tested)	ECV (μg/ml)	No. of isolates by ECV category (no. with acquired resistance mutation[s])	
			≤ECV	>ECV
C. albicans	Fluconazole (65)	0.5	18	47 (47)
	Posaconazole (65)	0.06	18 (5)	42 (42)
	Voriconazole (65)	0.03	18 (3)	44 (44)
C. dubliniensis	Fluconazole (38)	0.5	12	26 (26)
	Posaconazole (4)	0.25	1 (2)	1 (1)
	Voriconazole (4)	0.03		4 (3)
C. glabrata	Fluconazole (89)	8	29	60 (57)
	Voriconazole (49)	0.25	12 (7)	30 (30)
C. parapsilosis	Fluconazole (4)	1	1	3 (3)
	Posaconazole (4)	0.25	1	3 (3)
	Voriconazole (4)	0.03	1	3 (3)
C. tropicalis	Fluconazole (4)	1	1	3 (3)
	Voriconazole (4)	0.06	1	3 (3)

[a] Data were compiled from references 6–8, 10, and 18–26. Azole resistance mechanisms included mutations in and overexpression of *ERG11* and/or overexpression of MDR or CDR efflux pumps.

conazole, and all the isolates were characterized regarding the presence (non-WT) or absence (WT) of azole resistance mechanisms (6–8, 10, 18–26). A total of 136 isolates harbored molecularly defined azole resistance mechanisms: 47 *C. albicans*, 26 *C. dubliniensis*, and 57 *C. glabrata* isolates and 3 isolates each of *C. parapsilosis* and *C. tropicalis* (Table 5). The ECVs for fluconazole and *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis* were 0.5 μg/ml, 0.5 μg/ml, 8 μg/ml, 1 μg/ml, and 1 μg/ml, respectively (Table 4). Using these fluconazole cutoffs, the CLSI method correctly classified all (100%) of the 136 strains with resistance mutations/mechanisms as non-WT (MIC > ECV) and 61 (95.3%) of the 64 strains with no demonstrated resistance mutations/mechanisms as WT strains. The ECVs for posaconazole and *C. albicans*, *C. dubliniensis*, and *C. parapsilosis* were 0.06 μg/ml, 0.25 μg/ml, and 0.25 μg/ml, respectively (Table 4). Using these ECVs, the CLSI method with posaconazole correctly classified 46 (86.8%) of 53 strains with resistance mutations/mechanisms as non-WT and all 20 (100.0%) WT strains (MIC < ECV). Although a total of 7 isolates (5 of *C. albicans* and 2 of *C. dubliniensis*) with molecularly defined resistance mechanisms were classified as WT for posaconazole, this may be explained by the fact that certain azole resistance mechanisms (e.g., substitutions and missense mutations in or upregulation of *ERG11* or *MDR1*) may affect fluconazole to a greater extent than other azoles (6, 18, 19); all 7 of these isolates were non-WT for fluconazole. The ECVs for voriconazole and *C. albicans*, *C. dubliniensis*, and *C. parapsilosis* were all 0.03 μg/ml, and those for *C. glabrata* and *C. tropicalis* were 0.25 μg/ml and 0.06 μg/ml, respectively (Table 4). Using these ECVs, the CLSI method when used for voriconazole correctly classified 83 (89.2%) of 93 strains with resistance mutations/mechanisms as non-WT and 32 (97%) of the 33 WT strains. As with posaconazole, all 10 of the strains with molecularly defined resistance mutations/mechanisms that were classified as WT for voriconazole were non-WT for fluconazole, reflecting more fluconazole-specific resistance mechanisms. These results support the ability of the triazole ECVs to differentiate WT strains of *Candida* spp. from those harboring clinically important resistance mechanisms; additional data for *C. parapsilosis* and *C. tropicalis* are needed.

ECVs for *C. krusei* and the less prevalent species *C. guilliermondii* and *C. lusitaniae* are also depicted in Table 4. Since MIC distributions encompassed less than 100 values, ECVs were not defined for the other four less prevalent species (*C. famata*, *C. kefyr*, *C. orthopsilosis*, and *C. rugosa*), but their MIC distributions (Tables 1 to 3) are provided so that they may serve as a reference for other studies using the CLSI method. As expected, the fluconazole ECV for *C. krusei* was higher (32 μg/ml) than those for *C. lusitaniae* and *C. guilliermondii* (1 and 8 μg/ml, respectively). ECVs for *C. guilliermondii*, *C. krusei*, and *C. lusitaniae* and the other two triazoles were 0.5, 0.5, and 0.06 μg/ml (for posaconazole) and 0.12, 0.25, and 0.06, μg/ml (for voriconazole), respectively. To our knowledge, information regarding mechanisms of resistance is only available for *C. krusei*, and resistance to fluconazole has been postulated to be due to either a decreased sensitivity of the target enzyme or target mutations of the efflux pumps (11, 39, 40). More recently, only a 2-fold decrease in the fluconazole MIC (32 to 8 μg/ml) was observed in 1 of the 21 isolates evaluated (fluconazole MICs ≥ 16 μg/ml) by using the efflux pump inhibitor carbonyl cyanide 3-chloro-phenylhydrazone; no changes were observed among the voriconazole MICs (range, 0.06 to 0.25 μg/ml) (37), and hence the results are inconclusive regarding the efflux pumps. Due to the innate resistance of *C. krusei* to fluconazole, the CLSI does not recommend the interpretation of MICs for this species and agent (13), a recommendation that should be extended to the ECV. The CLSI and EUCAST susceptibility BP for *C. krusei* and voriconazole is ≤0.5 μg/ml (versus our ECV of 0.25 μg/ml); the EUCAST posaconazole ECV for *C. guilliermondii* is 0.25 μg/ml (versus our ECV of 0.5 μg/ml) (Table 4) (13, 29, 41).

In conclusion, we have defined ECVs for 8 of the 12 *Candida* spp. evaluated and the three triazoles (fluconazole ECVs ranged from 0.5 μg/ml for *C. albicans* and *C. dubliniensis* to 32 μg/ml for *C. krusei*, posaconazole ECVs ranged from 0.06 μg/ml for *C. albicans* and *C. lusitaniae* to 1 μg/ml for *C. glabrata*, and voriconazole ECVs ranged from 0.03 μg/ml for *C. albicans*, *C. dubliniensis*, and *C. parapsilosis* to 0.25 μg/ml for *C. glabrata* and *C. krusei*). These ECVs encompass 95% of the statistically modeled population and will serve to differentiate WT from non-WT strains of *Candida* for the three systemically active triazoles. We have demonstrated the ability of the species-specific ECVs for all three triazoles to identify those strains of *Candida* spp. harboring azole resistance mechanisms in a population of 200 well-characterized *Candida* species. The ECVs for fluconazole, posaconazole, and voriconazole and the CLSI broth microdilution method will help in monitoring the emergence of azole resistance among target species of *Candida*.

ACKNOWLEDGMENTS

L. Ostrosky-Zeichner has received research grants from and is a consultant and/or speaker for Pfizer, Merck, and Astellas.

The findings and conclusions of this article are those of the authors

and do not necessarily represent the views of the Centers for Disease Control and Prevention.

REFERENCES

1. **Messer SA, Jones RN, Fritsche TR.** 2006. International surveillance of *Candida* spp. and *Aspergillus* spp.: report from the SENTRY Antimicrobial Surveillance Program (2003). J. Clin. Microbiol. **44:**1782–1787. http://dx.doi.org/10.1128/JCM.44.5.1782-1787.2006.
2. **Costa-de-Oliveira S, Pina-Vaz C, Mendonca D, Goncalves Rodrigues A.** 2008. A first Portuguese epidemiological survey of fungaemia in a university hospital. Eur. J. Clin. Microbiol. Infect. Dis. **27:**365–374. http://dx.doi.org/10.1007/s10096-007-0448-4.
3. **Pappas PG, Kauffman CA, Andes D, Benjamin DK, Jr, Calandra TF, Edwards JE, Filler SG, Fischer JF, Kullberg BJ, Ostrosky-Zeichner L, Reboli AC, Rex JH, Walsh TJ, Sobel JD.** 2009. Clinical practice guidelines for the management of candidiasis: 2009 update by the Infectious Diseases Society of America. Clin. Infect. Dis. **48:**503–535. http://dx.doi.org/10.1086/596757.
4. **Cornely OA, Bassetti M, Calandra T, Garbino J, Kullberg BJ, Lortholary O, Meersseman W, Akova M, Arendrup MC, Arikan-Akdagli S, Bille J, Castagnola E, Cuenca-Estrella M, Donnelly JP, Groll AH, Herbrecht R, Hope WW, Jensen HE, Lass-Flörl C, Petrikkos G, Richardson MD, Roilides E, Verweij PE, Viscoli C, Ullmann AJ, ESCMID Fungal Infection Study Group.** 2012. ESCMID guideline for the diagnosis and management of *Candida* diseases 2012: non-neutropenic adult patients. Clin. Microbiol. Infect. **18:**19–37. http://dx.doi.org/10.1111/1469-0691.12039.
5. **Espinel-Ingroff A, Pfaller M, Canton E, Peman J.** 2010. Emerging resistance to azoles and echinocandins: clinical relevance and laboratory detection. Curr. Fungal Infect. Rep. **4:**186–195. http://dx.doi.org/10.1007/s12281-010-0026-6.
6. **Chau AS, Mendrick CA, Sabatelli FJ, Loebenberg D, McNicholas PM.** 2004. Application of real-time quantitative PCR to molecular analysis of *Candida albicans* strains exhibiting reduced susceptibility to azoles. Antimicrob. Agents Chemother. **48:**2124–2131. http://dx.doi.org/10.1128/AAC.48.6.2124-2131.2004.
7. **Sanglard D, Ischer F, Calabrese D, Majcherczyk PA, Bille J.** 1999. The ATP binding cassette transporter gene *CgCDR1* from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. Antimicrob. Agents Chemother. **43:**2753–2765.
8. **Silva AP, Miranda IM, Guida A, Synnott J, Rocha R, Silva R, Amorim A, Pina-Vaz C, Butler G, Rodrigues AG.** 2011. Transcriptional profiling of azole-resistant *Candida parapsilosis* strains. Antimicrob. Agents Chemother. **55:**3546–3556. http://dx.doi.org/10.1128/AAC.01127-10.
9. **Vandeputte P, Larcher G, Berges T, Renier G, Chabasse D, Bouchara J-P.** 2005. Mechanisms of azole resistance in a clinical isolate of *Candida tropicalis*. Antimicrob. Agents Chemother. **49:**4608–4615. http://dx.doi.org/10.1128/AAC.49.11.4608-4615.2005.
10. **Moran GP, Sanglard D, Donnelly SM, Shanley DB, Sullivan DJ, Coleman DC.** 1998. Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. Antimicrob. Agents Chemother. **42:**1819–1830.
11. **Orozco AS, Higginbotham LM, Hitchcock CA, Parkinson T, Falconer D, Ibrahim AS, Ghannoum MA, Filler SG.** 1998. Mechanism of fluconazole resistance in *Candida krusei*. Antimicrob. Agents Chemother. **42:**2645–2649.
12. **Pfaller MA, Diekema DJ.** 2012. Progress in antifungal susceptibility testing of *Candida* spp. by use of Clinical and Laboratory Standards Institute broth microdilution methods, 2010 to 2012. J. Clin. Microbiol. **50:**2846–2856. http://dx.doi.org/10.1128/JCM.00937-12.
13. **Clinical and Laboratory Standards Institute.** 2012. M27-S4. Reference method for broth dilution antifungal susceptibility testing of yeasts, 4th informational supplement. Clinical and Laboratory Standards Institute, Wayne, PA.
14. **Kahlmeter G, Brown DF, Goldstein FW, MacGowan AP, Mouton JW, Osterlund A, Rodloff A, Steinbakk M, Urbaskova P, Vatopoulos A.** 2003. European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. J. Antimicrob. Chemother. **52:**145–148. http://dx.doi.org/10.1093/jac/dkg312.
15. **Simjee S, Silley P, Werling HO, Bywater R.** 2008. Potential confusion regarding the term "resistance" in epidemiological surveys. J. Antimicrob. Chemother. **61:**228–229. http://dx.doi.org/10.1093/jac/dkm423.
16. **Turnidge J, Patterson DL.** 2007. Setting and revising antibacterial susceptibility breakpoints. Clin. Microbiol. Rev. **20:**391–408. http://dx.doi.org/10.1128/CMR.00047-06.
17. **Clinical and Laboratory Standards Institute.** 2008. M27-A3. Reference method for broth dilution antifungal susceptibility testing of yeasts, 3rd ed. Clinical and Laboratory Standards Institute, Wayne, PA.
18. **Xiao L, Madison V, Chau AS, Loebenberg D, Palermo RF, McNicholas PM.** 2004. Three-dimensional models of wild-type and mutated forms of cytochrome P450 14-alpha-sterol demethylases from *Aspergillus fumigatus* and *Candida albicans* provide insights into posaconazole binding. Antimicrob. Agents Chemother. **48:**568–574. http://dx.doi.org/10.1128/AAC.48.2.568-574.2004.
19. **MacCallum DM, Coste A, Ischer F, Jacobsen MD, Odds FC, Sanglard D.** 2010. Genetic sissection of azole resistance mechanisms in *Candida albicans* and their validation in a mouse model of disseminated infection. Antimicrob. Agents Chemother. **54:**1476–11483. http://dx.doi.org/10.1128/AAC.01645-09.
20. **Pinjon E, Moran GP, Jackson CJ, Kelly SL, Sanglard D, Coleman DC, Sullivan DJ.** 2003. Molecular mechanisms of itraconazole resistance in *Candida dubliniensis*. Antimicrob. Agents Chemother. **47:**2424–2437. http://dx.doi.org/10.1128/AAC.47.8.2424-2437.2003.
21. **Pinjon E, Jackson CJ, Kelly SL, Sanglard D, Moran G, Sullivan DJ.** 2005. Reduced azole susceptibility in genotype 3 *Candida dubliniensis* isolates associated with increased *CdCDR1* and *CdCDR2* expression. Antimicrob. Agents Chemother. **49:**1312–1318. http://dx.doi.org/10.1128/AAC.49.4.1312-1318.2005.
22. **Sanguinetti M, Posteraro B, Fiori B, Ranno S, Torelli R, Fadda G.** 2005. Mechanisms of azole resistance in clinical isolates of *Candida glabrata* collected during a hospital survey of antifungal resistance. Antimicrob. Agents Chemother. **49:**668–679. http://dx.doi.org/10.1128/AAC.49.2.668-679.2005.
23. **Borst A, Raimer MT, Warnock DW, Morrison CJ, Arthington-Skaggs BA.** 2005. Rapid acquisition of stable azole resistance by *Candida glabrata* isolates obtained before the clinical introduction of fluconazole. Antimicrob. Agents Chemother. **49:**783–787. http://dx.doi.org/10.1128/AAC.49.2.783-787.2005.
24. **Posteraro B, Tumbarello M, La Sorda M, Spanu T, Trecarichi EM, De Bernardis F, Scoppettuolo G, Sanguinetti M, Fadda G.** 2006. Azole resistance of *Candida glabrata* in a case of recurrent fungemia. J. Clin. Microb. **44:**3046–3047. http://dx.doi.org/10.1128/JCM.00526-06.
25. **Ferrari S, Ischer F, Calabrese D, Posteraro B, Sanguinetti M, Fadda G, Rohde B, Bauser C, Bader O, Sanglard D.** 2009. Gain of function mutations in *CgPDR1* of *Candida glabrata* not only mediate antifungal resistance but also enhance virulence. PLoS Pathog. **5:**e1000268. http://dx.doi.org/10.1371/journal.ppat.1000268.
26. **Forastiero A, Mesa-Arango AC, Alastruey-Izquierdo A, Alcazar-Fuoli L, Bernal Martinez L, Pelaez T, Lopez JF, Grimalt JO, Gomez-Lopez A, Cuesta I, Zaragoza O, Mellado E.** 2013. *Candida tropicalis* antifungal cross-resistance is related to different azole target (Erg11p) modifications. Antimicrob. Agents Chemother. **57:**4769–4781. http://dx.doi.org/10.1128/AAC.00477-13.
27. **Howell SA, Hazen KC.** 2011. *Candida*, *Cryptococcus*, and other yeasts of medical importance: mycology, p 1793–1821. *In* Versalovic J, Carroll KC, Jorgensen JH, Funke G, Landry ML, Warnock DW (ed), Manual of clinical microbiology, 10th ed, vol 2. ASM Press, Washington, DC.
28. **Turnidge J, Kahmeter G, Kronvall G.** 2006. Statistical characterization of bacterial wild-type MIC value distributions and determination of epidemiological cutoff values. Clin. Microbiol. Infect. **12:**418–425. http://dx.doi.org/10.1111/j.1469-0691.2006.01377.x.
29. **Espinel-Ingroff A, Cuenca-Estrella M, Canton E.** 2013. CLSI and EUCAST: working together towards a harmonized method for antifungal susceptibility testing. Curr. Fungal Infect. Rep. **7:**59–67. http://dx.doi.org/10.1007/s12281-012-0125-7.
30. **Espinel-Ingroff A, Chowdhary A, Gonzalez GM, Lass-Flörl C, Martin-Mazuelos E, Meis J, Pelaez T, Pfaller MA, Turnidge.** 2013. J. Multicenter study of isavuconazole MIC distributions and epidemiological cutoff values for *Aspergillus* spp. for the CLSI M38-A2 broth microdilution method. Antimicrob. Agents Chemother. **57:**3823–3828. http://dx.doi.org/10.1128/AAC.00636-13.
31. **Espinel-Ingroff A, Arendrup MC, Pfaller MA, Bonfietti LX, Bustamante B, Canton E, Chryssanthou E, Cuenca-Estrella M, Dannaoui E, Fothergill A, Fuller J, Gaustad P, Gonzalez GM, Guarro J, Lass-Flörl C, Lockhart SR, Meis JF, Moore CB, Ostrosky-Zeichner L, Pelaez T,**

Pukinskas SRBS, St-Germain G, Szeszs MW, Turnidge J. 2013. Inter-laboratory variability of caspofungin MICs for *Candida* spp. using CLSI and EUCAST methods: should the clinical laboratory be testing this agent? Antimicrob. Agents Chemother. **57:**5836–5842. http://dx.doi.org/10.1128/AAC.01519-13.

32. **Pfaller MA, Espinel-Ingroff A, Bustamante B, Canton E, Diekema DJ, Fothergill A, Fuller J, Gonzalez GM, Guarro J, Lass-Flörl C, Lockhart SR, Martin-Mazuelos E, Meis JF, Ostrosky-Zeichner L, Pelaez T, St-Germain G, Turnidge J.** 2014. Multicenter study of anidulafungin and micafungin MIC distributions and epidemiological cutoff values for eight *Candida* species and the CLSI M27-A3 broth microdilution method. Antimicrob. Agents Chemother. **58:**916–922. http://dx.doi.org/10.1128/AAC.02020-13.
33. **Johnson E, Espinel-Ingroff A, Szekely A, Hockey H, Troke P.** 2008. Activity of voriconazole, itraconazole, fluconazole and amphotericin B against 1763 yeasts from 472 patients in the voriconazole phase III clinical studies. Int. J. Antimicrob. Agents **32:**511–514. http://dx.doi.org/10.1016/j.ijantimicag.2008.05.023.
34. **Sabatelli F, Patel R, Mann PA, Mendrick CA, Norris CC, Hare R, Loebenberg D, Black TA, McNicholas PM.** 2006. *In vitro* activity of posaconazole, fluconazole, itraconazole, voriconazole, and amphotericin B against a large collection of clinically important molds and yeasts. Antimicrob. Agents Chemother. **50:**2009–2015. http://dx.doi.org/10.1128/AAC.00163-06.
35. **Pfaller MA, Messer SA, Gee S, Joly S, Pujol C, Sullivan DJ, Coleman DC, Soll DR.** 1999. *In vitro* susceptibilities of *Candida dubliniensis* isolates tested against the new triazole and echinocandin antifungal agents. J. Clin. Microbiol. **37:**870–872.
36. **Espinel-Ingroff A.** 2003. *In vitro* antifungal activities of anidulafungin and micafungin, licensed agents and the investigational triazole posaconazole as determined by NCCLS methods for 12,052 fungal isolates: review of the literature. Rev. Iberoam. Micol. **20:**121–136.
37. **Guinea J, Sanchez-Somolinos M, Cuevas O, Pelaez T, Bouza E.** 2006. Mechanism of fluconazole resistance mechanisms in *Candida krusei*: the contribution of efflux-pumps. Med. Mycol. **44:**575–579. http://dx.doi.org/10.1080/13693780600561544.
38. **Cantón E, Iñiguez C, Pemán J, Hervás D, Lopez-Hontangas JL, Pina-Vaz C, Camarena JJ, Campos-Herrero I, García-García I, García-Tapia AM, Guna R, Merino P, Pérez del Molino L, Rubio C, Suarez A, FUNGEMYCA Study Group.** 2013. Epidemiological cutoff values for fluconazole, itraconazole, posaconazole, and voriconazole for six *Candida* species as determined by the colorimetric Sensititre YeastOne method. J. Clin. Microbiol. **51:**2691–2695. http://dx.doi.org/10.1128/JCM.01230-13.
39. **Vanden Bossche H, Marichal P, Odds FC.** 1994. Molecular mechanisms of drug resistance in fungi. Trends Microbiol. **2:**393–400. http://dx.doi.org/10.1016/0966-842X(94)90618-1.
40. **Katiyar SK, Edlind TD.** 2001. Identification and expression of multidrug resistance related to ABC transporter genes in *Candida krusei*. Med. Mycol. **39:**109–116. http://dx.doi.org/10.1080/mmy.39.1.109.116, http://dx.doi.org/10.1080/714030988.
41. **Arendrup MC, Cuenca-Estrella M, Donnelly JP, the European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing.** 2011. EUCAST technical note on posaconazole. Clin. Microbiol. Infect. **17:**E16–E17. http://dx.doi.org/10.1111/j.1469-0691.2011.03646.x.