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SUSCEPTIBILITY

Etest ECVs/ECOFFs for Detection of Resistance in Prevalent and Three Nonprevalent *Candida* spp. to Triazoles and Amphotericin B and *Aspergillus* spp. to Caspofungin: Further Assessment of Modal Variability

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ABSTRACT Susceptibility testing is an important tool in the clinical setting; its utility is based on the availability of categorical endpoints, breakpoints (BPs), or epidemiological cutoff values (ECVs/ECOFFs). CLSI and EUCAST have developed antifungal susceptibility testing, BPs, and ECVs for some fungal species. Although the concentration gradient strip bioMérieux Etest is useful for routine testing in the clinical laboratory, ECVs are not available for all agent/species; the lack of clinical data precludes development of BPs. We reevaluated and consolidated Etest data points from three previous studies and included

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new data. We defined ECOFFinder Etest ECVs for three sets of species-agent combinations: fluconazole, posaconazole, and voriconazole and 9 *Candida* spp.; amphotericin B and 3 nonprevalent *Candida* spp.; and caspofungin and 4 *Aspergillus* spp. The total of Etest MICs from 23 laboratories (Europe, the Americas, and South Africa) included (antifungal agent dependent): 17,242 *Candida albicans*, 244 *C. dubliniensis*, 5,129 *C. glabrata* species complex (SC), 275 *C. guilliermondii* (*Meyerozyma guilliermondii*), 1,133 *C. krusei* (*Pichia kudriavzevii*), 933 *C. kefyr* (*Kluyveromyces marxianus*), 519 *C. lusitaniae* (*Clavispora lusitaniae*), 2,947 *C. parapsilosis* SC, 2,214 *C. tropicalis*, 3,212 *Aspergillus fumigatus*, 232 *A. flavus*, 181 *A. niger*, and 267 *A. terreus* SC isolates. Triazole MICs for 66 confirmed non-wild-type (non-WT) *Candida* isolates were available (*ERG11* point mutations). Distributions fulfilling CLSI ECV criteria were pooled, and ECOFFinder Etest ECVs were established for triazoles (9 *Candida* spp.), amphotericin B (3 less-prevalent *Candida* spp.), and caspofungin (4 *Aspergillus* spp.). Etest fluconazole ECVs could be good detectors of *Candida* non-WT isolates (59/61 non-WT, 4 of 6 species).

KEYWORDS *Aspergillus* spp., *Candida*, ECOFFS, ECVs, ERG11 mutants, amphotericin B, antifungal resistance, caspofungin, nonprevalent *Candida*, triazoles

The most common fungal species causing serious infections are *Candida albicans* and *Aspergillus fumigatus*. Recently, other common and less prevalent fungal species have emerged as important pathogens, especially in the immunocompromised host and those patients with serious underlying diseases (1–6). The attributable mortality rate among patients suffering invasive fungal infections can be as high as 47%, depending on the patient population and age (1). The polyenes, triazoles, and echinocandins are the current treatments for severe/invasive candidiasis and aspergillosis (3–6). The determination of MICs/MECs (MICs/minimal effective concentrations) is recommended for isolates from bloodstream infections or any other sterile sites or for *Aspergillus/Candida* isolates recovered from patients with other severe infections. In the case of initial infections, susceptibility testing is only recommended in areas of high resistance prevalence (2, 4, 7).

MICs/MECs can be determined for yeasts and molds by either the Clinical and Laboratory Standards Institute (CLSI) and/or European Committee on Antimicrobial Susceptibility Testing (EUCAST) methods. For antifungal susceptibility testing to be useful in the clinical setting, the results should be comparable in the different laboratories, and method-dependent interpretive criteria ought to be available to categorize the MIC/MEC results (8–12). Breakpoints (BPs) and epidemiological cutoff values (ECVs/ECOFFs) are available for these reference methods, especially the latter, for a variety of mold and yeast species (8–12). Several commercial methods are frequently used in clinical and research laboratories; among them, we are reporting >95% MIC data by the concentration gradient strip bioMérieux Etest method (Etest) (13–16). The lack of clinical data precludes the establishment of BPs for commercial methods. However, ECVs have been defined based solely on *in vitro* data, including those for the Etest and some species-agent combinations (17–19). ECVs could be useful in the surveillance of *in vitro* resistance and to distinguish between phenotypic wild-type (WT; no detectable resistance) and non-WT (mutants or isolates having known molecular mechanisms) isolates. The latter are less likely to respond to contemporary therapy (8, 10, 12). When limited clinical data have precluded the development of BPs for the method being utilized, the role of the ECV is to (i) predict the likely outcome of antifungal therapy and guide therapy in specific clinical circumstances, (ii) detect resistance development during therapy, and (iii) monitor local susceptibility patterns that will guide empirical therapy (8, 10, 12).

Etest ECVs for the triazoles (fluconazole, posaconazole, and voriconazole) and *Candida* spp. were calculated in two consecutive collaborative studies (17, 18). However, in the first study, modal variability among the participant laboratories or insufficient data precluded ECV definition for various *Candida* species, especially fluconazole ECVs (17). In addition, the ECVs calculated in these two studies were different for various species. In the second study, Etest

amphotericin B ECVs for three less prevalent *Candida* spp. (*C. guilliermondii* [*Meyerozyma guilliermondii*], *C. kefyr* [*Kluyveromyces marxianus*], and *C. lusitaniae* [*Clavispora lusitaniae*]) as well as caspofungin ECVs for *A. fumigatus* species complex (SC) were also reported (18); from this point the most common names will be used. Owing to modal variability among Etest caspofungin MIC distributions for *Aspergillus* spp., ECVs were not defined in a third study (19). It is interesting that modal variability among CLSI and EUCAST caspofungin MICs also precluded the definition of reference ECVs for *Candida* spp. but not for *Aspergillus* spp. (20, 21). These conflicting results required a modal reevaluation of the available Etest data by consolidating those data points as single species/agent distributions for ECV definition (17–19). In the last few years, an increase in the incidence of less prevalent *Candida* spp. has been evident among cancer and other immunocompromised patients, and large numbers of isolates were included in the *in vitro* studies of the antifungal agents under development (22–24). Therefore, we were able to add sufficient amphotericin B, triazole, and caspofungin MICs for our calculation of Etest ECVs for *Candida* (including those three less prevalent species) and *Aspergillus* species evaluated in the present study.

The main purpose of the present study was to consolidate and reanalyze Etest data reported in three published studies to define and update triazole and amphotericin B ECVs for *Candida* spp. and caspofungin ECVs for *Aspergillus* spp. (17–19); substantial additional data points also were evaluated. Therefore, we pooled (i) fluconazole, posaconazole, and voriconazole MIC distributions for five prevalent and four less common *Candida* spp., (ii) amphotericin B MIC distributions for the same less prevalent *Candida* spp., and (iii) caspofungin MICs for four *Aspergillus* SC. The pooled Etest MIC distributions were then analyzed, including the interlaboratory modal agreement (modes within one 2-fold dilution), and proposed ECOFFinder Etest ECVs for triazoles and amphotericin B (*Candida* spp.) and caspofungin (*Aspergillus* spp.) (25). Each individual distribution included ≥100 MIC values that originated in 3 to 23 independent laboratories (CLSI criteria) (8, 25). ECVs were considered tentative when they were based on data from only 3 to 4 laboratories or the particular distributions were bimodal.

Knowledge about genetic resistance mechanisms among *Candida* spp. (triazoles and echinocandins) is important, since antifungal resistance continues to spread, including multidrug resistance (1, 26–31). As most of the isolates included in the study were not assessed for mechanisms of resistance, we evaluated the application of our triazole ECVs against Etest MIC data for individual and well-characterized non-WT isolates harboring *ERG11* gene point mutations or *CDR2* and *MDR* overexpression as previously reported in other studies (17, 19, 26, 28, 30, 31). The set of 66 mutants included 6 *C. albicans*, 5 *C. glabrata*, 2 *C. guilliermondii*, 2 *C. krusei*, 45 *C. parapsilosis*, and 6 *C. tropicalis*.

RESULTS AND DISCUSSION

BPs are unique and reliable predictors of clinical response to therapy for the isolate/agent being evaluated for triazoles and echinocandins and certain *Candida* spp. EUCAST has also developed amphotericin B BPs (11, 12). CLSI/EUCAST have established ECVs for a variety of yeast and mold species based solely on MIC/MEC results (9, 10, 12). By definition, ECVs categorize isolates as either WT (no detectable phenotypic resistance) or non-WT (an isolate that could be harboring acquired resistance mechanisms) (8, 10, 12, 25). Commercial methods, including the Etest, use reference endpoints as predictors of antifungal resistance (non-WT or resistant isolates) (14). Classification of an isolate as non-WT indicates that it has "reduced susceptibility to the agent being evaluated" (8). There is no need to know the putative resistance mechanism in order to classify an isolate as non-WT; it is also possible that an isolate harboring a known resistance mechanism could be classified as WT as reported below, especially when there is an overlap of the MICs of strains with acquired resistance mechanisms at the upper end of the WT MIC distribution (12, 25). We believe that the Etest ECVs reported in this paper could better identify potential acquired resistance instead of relying on reference interpretive endpoints, e.g., CLSI BPs (14). The reason for that is that both ECVs and BPs are method dependent, and isolates should be categorized by the endpoints of the method used to produce the MIC result

(8, 10). In general, ECVs also could be suitable for resistance surveillance or epidemiological purposes as well as being a convenient screening method. It is important to categorize relevant strains recovered from sterile body sites, including tissue biopsy specimens, bone and fluids, cerebrospinal, peritoneal, pericardial, pleural, and synovial fluids, among others (2, 3, 7).

The total triazole Etest data evaluated and originating in Europe, the Americas, and South Africa were the following (17–19): (i) 17,242 *Candida albicans*, 244 *C. dubliniensis*, 5,129 *C. glabrata* species complex [SC], 1,133 *C. krusei*, 2,947 *C. parapsilosis* SC, 2,214 *C. tropicalis*, 275 *C. guilliermondii*, 933 *C. kefyr*, and 519 *C. lusitaniae* (17, 18); (ii) Etest amphotericin B MICs for 190 *C. guilliermondii*, 481 *C. kefyr*, and 330 *C. lusitaniae*; and (iii) caspofungin MICs were analyzed for 3,212 *A. fumigatus* SC (belonging to the section *Fumigati*), 232 *A. flavus* SC (belonging to the section *Flavi*), 181 *A. niger* SC (belonging to the section *Nigri*), and 267 *A. terreus* SC (belonging to the section *Terrei*). Our ECVs were defined by the CLSI criteria as summarized elsewhere (8, 10). We met those criteria for the minimum of 100 MIC values for each pooled nonmutant distribution in our ECOFFinder ECV analysis (each agent-species combination) (8, 25). The criteria regarding the number of isolates in each single distribution before pooling was $<$50%, with three exceptions (caspofungin data for the three species of non-*A. fumigatus*). Therefore, only those three sets of data were weighed prior to the ECOFFinder analysis, and the resulting ECVs were the same. Etest MICs were submitted for 66 *ERG11* mutants from five single laboratories. Given that Etest BPs are not available for these antifungal agent and fungal species combinations, the ECVs proposed in the present study could help the clinician and laboratory personnel in identifying isolates with possible acquired resistance mechanisms. Etest data points were only included in the study when the MIC ranges were within the MIC range for the QC isolates.

Etest ECVs for triazoles and *Candida* spp. The Etest triazole MIC distributions for the *Candida* spp. evaluated are depicted in Table 1. The number of fluconazole MICs ranged from 17,242 to 244 data points from 3 to 20 laboratories, including those for the species complexes of *C. glabrata* and *C. parapsilosis* and the less prevalent species, *C. kefyr*, *C. guilliermondii*, *C. lusitaniae*, and *C. dubliniensis*. The available voriconazole and posaconazole data points were smaller: acceptable voriconazole MIC counts ranged from 10,806 to 275 evaluated in 11 to 19 laboratories for 8 species, while posaconazole data ranged from 2,085 to 162 Etest MIC counts from 4 to 14 laboratories for four of the prevalent species. Although Etest MICs for the species evaluated originated from 23 participant centers, the following exclusions were made after the review of individual distributions: (i) when the particular mode for a distribution was more than 1 to 2 dilutions from the most common mode; (ii) when the mode was not clearly defined; or (iii) where the distribution was truncated within the putative wild-type population. These criteria were modified from those previously described (8, 10) in order to capture as much useful data as possible on less common species.

The summary of fluconazole and voriconazole Etest ECOFFinder ECVs for 8 to 9 *Candida* spp. is depicted in Table 2 (including the two tentative ECVs discussed below). Only three posaconazole ECVs were calculated; for the fourth species (*C. glabrata*), the aggregated WT distribution was asymmetric, which precluded ECV estimation. The asymmetry was due to the high proportions of non-wild-type MICs from some laboratories. Although similar asymmetry was also observed among both fluconazole and voriconazole MIC distributions for *C. glabrata*, sufficient data points allowed us to define tentative fluconazole and voriconazole ECVs for this species (Tables 1 and 2). It is interesting that the analysis of the combined asymmetric and nonasymmetric distributions provided the same ECV.

We did not attempt to compare all the ECV values in Table 2 with those defined in the two previous and related studies (17, 18). One of the reasons is that, in many instances, ECVs were not defined for some of the species in both studies. In one of the two previous studies, Etest fluconazole and voriconazole ECVs for *C. glabrata* were 64 μg/ml and 2 μg/ml, respectively (17), but they were not calculated in the second

TABLE 1 Pooled MIC distributions of three azoles for prevalent and nonprevalent *Candida* spp. determined by the commercial Etest method[a]

Species[b]	No. of labs	No. of isolates	No. of isolates with MIC (μg/ml) of[c]																
			≤0.002	0.004	0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	≥256
Fluconazole																				
C. albicans	18	17,242				27	94	758	4,362	**7,226**	3,138	948	318	109	38	47	27	18	12	120
C. glabrata	20	5,129				4	2	3	9	17	39	93	215	433	709	**1,199**	927	385	147	947
C. parapsilosis	20	2,947				4	16	51	236	663	**866**	627	244	72	39	32	13	7	5	72
C. tropicalis	19	2,214				2	4	9	34	200	619	**772**	378	79	35	20	16	11	2	33
C. krusei[d]	15	1,122									4	4	5	14	51	188	260	62	31	**405**
C. kefyr	14	933				1	10	71	239	**360**	204	28	8	1	3	1	1		1	6
C. lusitaniae	15	519				5	11	30	90	**159**	115	46	16	3	5	5	10	6	3	15
C. guilliermondii	9	257								3	14	39	58	**61**	36	16	7	8	4	11
C. dubliniensis	3	244				2	2	37	**92**	63	28	7	8	3			2			
Posaconazole																				
C. albicans	14	2,085	7	50	348	**846**	575	157	47	19	15	11	2	1			7[e]			
C. glabrata[d]	10	740			4	6	5	15	22	31	68	105	91	86	44	37	**226**			
C. tropicalis	11	384	2		22	67	**119**	88	40	29	8	4	2		2		1[e]			
C. parapsilosis	4	162	1	7	26	**51**	37	23	9	3	2			1			2[e]			
Voriconazole																				
C. albicans	19	10,806	522	2,544	**3,988**	2,355	795	280	124	63	43	20	11	10	2	5	44[e]			
C. glabrata[d]	16	3,514		9	14	36	112	349	780	**786**	446	204	135	140	92	121	290[e]			
C. parapsilosis	14	1,674	20	101	237	358	**382**	287	152	58	25	16	21	12	1		4[e]			
C. tropicalis	18	1,639	3	10	27	168	407	**538**	334	66	43	16	7	4	3	3	9[e]			
C. krusei	16	1,133		1	1	4	11	64	197	**390**	340	81	24	12	4	2	2[e]			
C. kefyr	16	672	38	105	**240**	206	61	9	4	3	4		1	1						
C. lusitaniae	13	355	34	74	**152**	54	11	6	10	6	6	1		1						
C. guilliermondii	11	275		1	4	4	47	**76**	58	29	16	13	8	3		2	14[e]			

[a]MICs determined by the commercial bioMérieux Etest method according to the manufacturer's instructions (13).
[b]*C. guilliermondii* (*Meyerozyma guilliermondii*), *C. kefyr* (*Kluyveromyces marxianus*), *C. krusei* (*Pichia kudriavzevii*), *C. lusitaniae* (*Clavispora lusitaniae*), and the complexes of *C. glabrata* and *C. parapsilosis*.
[c]The highest number in each row (showing the most frequently obtained MIC or the mode) is in boldface.
[d]ECV was not defined for posaconazole and *C. glabrata* due to asymmetry in the aggregated MIC distribution.
[e]Values ≥32 μg/ml.

TABLE 2 Method-dependent Etest ECOFFinder ECVs of three triazoles and amphotericin B for prevalent and nonprevalent species of *Candida*[a]

Agent and species[b]	No. of isolates tested	No. of labs with acceptable data	MIC (μg/ml)		ECVs (μg/ml),[c] 95%/97.5%
			Observed range	Wild-type mode	
Fluconazole					
C. albicans	17,242	18	0.016–>256	0.25	0.5/1
C. glabrata	1,334	13	0.016–>256	16	64/128[d]
C. parapsilosis	2,947	17	0.016–>256	0.5	2/2
C. tropicalis	2,214	19	0.016–>256	1	2/4
C. krusei	1,122	15	0.5–>256	32	128/128
C. kefyr	933	14	0.25–>256	0.25	0.5/1
C. lusitaniae	519	15	0.016–>256	0.25	1/2
C. guilliermondii	257	9	0.25–>256	4	16/16
C. dubliniensis	244	3	0.016–32	0.125	0.5/0.5[e]
Posaconazole					
C. albicans	2,085	14	≤0.002–>32	0.016	0.06/0.06
C. tropicalis	384	11	≤0.002–>32	0.03	0.25/0.25
C. parapsilosis	162	4	≤0.002–32	0.016	0.06/0.125[e]
Voriconazole					
C. albicans	10,806	19	≤0.002–>32	0.008	0.03/0.03
C. glabrata	2,333	13	0.004–>32	0.12	1/1[d]
C. krusei	1,133	16	0.004–>32	0.25	1/1
C. parapsilosis	1,674	14	≤0.002–>32	0.03	0.125/0.25
C. tropicalis	1,639	18	≤0.002–>32	0.06	0.25/0.25
C. guilliermondii	275	11	0.004–>32	0.06	0.25/0.5
C. kefyr	672	16	≤0.002–4	0.008	0.03/0.03
C. lusitaniae	355	13	≤0.002–4	0.008	0.016/0.03
Amphotericin B					
C. lusitaniae	330	13	0.008–4	0.25	1/1
C. kefyr	481	13	0.008–16	0.5	1/1
C. guilliermondii	190	7	0.016–16	0.25	1/1

[a]Including the complexes of *C. parapsilosis* and *C. glabrata*. Variability or insufficient data precluded the proposal of ECVs as the following: aberrant mode and no reliable mode (e.g., fluconazole and *C. albicans* and posaconazole and *C. glabrata*).
[b]*C. guilliermondii* (*Meyerozyma guilliermondii*), *C. kefyr* (*Kluyveromyces marxianus*), *C. krusei* (*Pichia kudriavzevii*), *C. lusitaniae* (*Clavispora lusitaniae*).
[c]ECVs for 95%/97.5% of the statistically modeled population by ECOFFinder calculations and based on MICs by the commercial bioMérieux Etest method (13).
[d]The calculated 95% value was 128 μg/ml but was reduced to 64 μg/ml due to some minor distortions in the data set; tentative values are shown.
[e]Tentative values, data are from only 3 to 4 laboratories.

study (18). Given that *C. krusei* is considered innately resistant to fluconazole, these isolates should be identified, but there is no need to perform fluconazole susceptibility testing; interpretative endpoints for this species are not listed any longer by the CLSI (9, 11). We have only reported an ECV for this species for future reference. Interpretation of the trailing growth is always difficult, especially after 48 h of incubation.

However, we increased the number of Etest triazole ECVs, and each ECV was defined with a higher number of isolates/species/agents (17, 18). We now have fluconazole and voriconazole ECVs for 9 and 8 *Candida* spp., respectively, instead of 2 to 7 (17, 18). The importance of using method-dependent ECVs is demonstrated when our Etest ECVs are compared to those by the two reference methods. Only fluconazole reference ECVs for *C. parapsilosis* and *C. dubliniensis* and posaconazole and voriconazole ECVs for *C. albicans* are the same as those by the Etest (Table 2) (9, 12). It is interesting that both the EUCAST and our Etest ECVs for *C. krusei* are the same (128 μg/ml), while the CLSI method does not list an ECV for this species (9).

The role of the ECV is to identify the strains that could harbor intrinsic or acquired resistance mechanisms (e.g., *Erg11* alterations or point mutations or other documented triazole resistance mechanisms in *Candida* spp.) (26–31). Although the literature has extensive reference MIC data for triazole mutants of *Candida*, that is not the case for the commercial methods, especially by the Etest (32, 33). A total of 66 Etest fluconazole MICs were received for *Candida* mutants, including 45 data points for *C. parapsilosis* (Table 3). However, only 17 posaconazole (not listed in Table 3) and 29 voriconazole MICs for mutants were evaluated. The *ERG11* mutations were the most

TABLE 3 Etest fluconazole and voriconazole MICs for 66 non-WT (mutant) *Candida* spp. isolates and ECVs[a]

Species[b] (no. of mutants)	FLU MICs	Etest ECV	VOR MIC	Etest ECVs	ERG point mutation(s)[c]
C. albicans (6)	8	1	2	0.03	A114S, G464S
	>128		1		11 T220L, E266D, 448R
	32		0.25		11 Y132H, T220L, V437I
	NG		0.5		11 F145T, T220L, V437I
	4		0.5		Y132F
	64		1		CDR2/MDR overexpression
C. glabrata (5)	128	128	2	1	A114S, G464S
	128		4		A114S, G464S
	128		4		A114S, G464S
	128		>8		A114S, G464S
	128		>8		A114S, G464S
C. guilliermondii (2)	16	16	0.25	0.5	A114S, G464S
	16		1		A114S, G464S
C. krusei (2)	64	16	>8	1	Y87X
	32		4		Y87X
C. parapsilosis (45)	4(2)	2	0.25	0.25	Y132F
	8(26)		ND		Y132F
	16(11)		0.25		Y132F
	32		0.25		A114S, G464S
	32		0.5		Y132F
	32		0.25		Y132F
	32		0.25		Y132F
	64		1		Y132F
	>64		0.25		Y132F
C. tropicalis (6)	>128	4	2	0.25	K143R
	>128		1		K143R
	>128		1		K143R
	>128		2		Y132F
	>128		>32		Y132F
	>128		>32		G464D

[a]Calculated ECVs/ECOFFS (epidemiological cutoff values) based on MICs determined by the commercial bioMérieux Etest method (13). Posaconacole data were not included, only three ECVs were defined. FLU, fluconazole; VOR, voriconazole.
[b]*C. guilliermondii* (*Meyerozyma guilliermondii*), *C. krusei* (*Pichia kudriavzevii*).
[c]*ERG11* gene amino acid point mutations; *CDR2* and *MDR* overexpression (29–31).

common mechanisms of acquired resistance to triazole agents encountered in the strains included in this work. Although the triazole MIC phenotype was not always altered, the fluconazole ECVs recognized 59 of the 61 *ERG11* mutants evaluated, not including the data for *C. glabrata* and *C. guilliermondii* (same or lower MIC value than the ECV). The same overlap also was observed with voriconazole (6/29) and especially with posaconazole (6/17) ECVs (posaconazole data are not listed in Table 3). Still, the number of mutants sought and identified was small. It would be interesting to apply our reevaluated Etest fluconazole and voriconazole ECVs against Etest MICs for a larger number of mutants harboring the same or other triazole resistance mechanisms.

Etest ECVs for amphotericin B and three less prevalent *Candida* spp. Antifungal susceptibility testing for amphotericin B and *Candida* spp. is problematic for many reasons. Genetic resistance mechanisms for this agent have not been clearly understood, and there is a lack of data from clinical trials. BPs are only available for fungal testing by the EUCAST for some of the common species (12). However, reference ECVs are available for a variety of species, including those for *C. lusitaniae*, *C. guilliermondii*, and *C. kefyr* (9). Our amphotericin B Etest MIC distributions for those three species are summarized in Table 4, and the Etest ECOFFinder ECVs are in Table 2. A total of 481 Etest MICs were pooled for *C. kefyr*, 330 for *C. lusitaniae*, and 190 for *C. guilliermondii* that

TABLE 4 Pooled MIC distributions of amphotericin B for three species of nonprevalent *Candida* spp. determined by the Etest method

Species[a]	No. of labs	No. of isolates tested	No. of isolates with MIC (μg/ml) of[b]										
			≤0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	>8
C. kefyr	13	481	1	3	10	23	45	129	**234**	31	4		1
C. lusitaniae	7	330	1	4	14	30	64	**123**	74	12	7	1	
C. guilliermondii	13	190		4	7	21	45	**60**	39	4	3	2	5

[a]*C. kefyr* (*Kluyveromyces marxianus*), *C. lusitaniae* (*Clavispora lusitaniae*), and *C. guilliermondii* (*Meyerozyma guilliermondii*).
[b]The largest number in each row (showing the most frequently obtained MIC or the mode) is in boldface. MICs were determined by the commercial bioMérieux Etest method (13).

originated in 7 and 13 laboratories. The amphotericin B 95% and 97.5% ECOFFinder ECVs were 1 μg/ml for each of these three species, but the mode was higher for *C. kefyr* (0.5 versus 0.25 μg/ml). The Etest MICs have been proposed in the literature as better predictors of therapeutic failure, especially for *C. lusitaniae* (16, 34). Among the data points for *C. lusitaniae*, we have included a set of 38 MICs for strains isolated from different patients reported as not having received any antifungal therapy (34). The reported Etest MIC range for these 38 *C. lusitaniae* isolates was 0.03 to 0.25 μg/ml. The Etest MIC range was 2 to 16 μg/ml and 0.5 to 2 μg/ml by the CLSI (16, 34) for three *C. lusitaniae* strains (codes 5W31, CL2819, and 2887) evaluated in animal studies, where mice were unresponsive to amphotericin B treatment. It is noteworthy that the current CLSI M59 document has the following notation: "Amphotericin B resistance is usually observed on agar assays instead of broth dilution methods" (9). Etest amphotericin B MICs for *C. lusitaniae* were low for the 330 isolates evaluated in our study (range, 0.01 to 4 μg/ml with a mode of 0.25 μg/ml) (Tables 2 and 4). It has been documented that amphotericin B resistance or increasing MICs can occur rapidly during therapy (e.g., amphotericin B EUCAST MICs from 0.25 to 2 μg/ml and Etest MICs from 0.25 to 1 μg/ml) (35). The EUCAST ECVs for these three species range from 0.5 to 1 μg/ml (12). Similar data were not found for the other two species.

Caspofungin Etest ECVs for four *Aspergillus* spp. Etest caspofungin MIC distributions for four common *Aspergillus* spp. are depicted in Table 5. As discussed above, an attempt was made to define ECVs for these species in a prior Etest study (19), but modal variability precluded the definition of Etest caspofungin ECVs for *Aspergillus* spp. The variability was mostly observed among the seven laboratories providing the data points for *A. fumigatus* at that time. In the present study, we were able to consolidate MIC data from 4 to 14 laboratories (both previous studies and additional data, including a set of 70 *A. fumigatus sensu stricto*). The total number of Etest caspofungin MICs ranged from 3,212 for *A. fumigatus* SC to 181 for *A. niger* SC, originating in 14 and 4 laboratories, respectively. The ECOFFinder 95 and 97.5% Etest caspofungin ECVs are depicted in Table 6; the ECVs were either 0.25 μg/ml or 0.5 μg/ml, with the exception of *A. terreus* SC (ECV, 2 μg/ml). Recently, echinocandin *in vitro* data were published for five molecularly analyzed *A. fumigatus* WT and three non-WT isolates, one of the latter a clinical isolate having a high CLSI echinocandin MEC in the absence of any *fks* mutations (36). The caspofungin MICs by the MTS brand gradient concentration strip agar method for the non-WT isolates (2 to 8 μg/ml) are above our Etest ECV of 0.25/ml for this species. The MIC range for the six WT isolates was 0.06 to 0.25 μg/ml (values equal

TABLE 5 Pooled MIC distributions of caspofungin for four species of *Aspergillus* determined by the commercial Etest method

Species[a]	No. of labs	No. of isolates	No. of isolates with MIC (μg/ml) of[b]										
			≤0.008	0.016	0.03	0.06	0.125	0.25	0.5	1	2	4	>8
A. fumigatus	14	3,212	93	333	905	**1,040**	598	208	18	4	1		12
A. flavus	6	232	5	20	42	**70**	70	18	4	2	1		
A. niger	4	181		8	15	**72**	72	13	1				
A. terreus	6	267	2	3	9	20	28	53	**114**	31	3		4

[a]Including the complexes of the four species of *Aspergillus* tested.
[b]The largest number in each row (showing the most frequently obtained MIC or the mode) is in boldface. MICs were obtained by the commercial bioMérieux Etest method (13).

TABLE 6 Method-dependent Etest ECOFFinder ECVs of caspofungin for four species of *Aspergillus*

Agent and species[a]	No. of isolates tested	No. of labs with acceptable data	MIC (μg/ml)		ECV (μg/ml),[b] 95%/97.5%
			Observed range	Wild-type mode	
A. fumigatus	3,212	14	0.008–>32	0.06	0.25/0.25
A. terreus	267	6	0.008–32	0.5	2/2
A. flavus	232	6	0.008–2	0.06	0.25/0.5
A. niger[c]	181	4	0.016–0.5	0.06	0.25/0.25

[a]Including the complexes of the four species tested.
[b]ECVs for 95%/97.5% of the statistically modeled population by ECOFFinder calculations and based on MICs by the commercial bioMérieux Etest method (13).
[c]Tentative value; data are from only 3 to 4 laboratories.

or below our ECV of 0.25 μg/ml). Reference MECs were comparable. To our knowledge, similar data are not found in the literature.

In conclusion, (i) including the two tentative ECVs for *C. glabrata*, we are providing Etest triazole ECVs for 9 (fluconazole), 8 (voriconazole), and 3 (posaconzole) *Candida* spp.; these ECVs were defined with an abundant or adequate number of isolates/species; (ii) we also added data points for amphotericin B and the three less common *Candida* spp.; and (iii) we were able to define caspofungin ECVs for four *Aspergillus* spp. This is important, since modal irregularities in previous studies among the participant laboratories or insufficient data have precluded the Etest ECV calculation for caspofungin and *Aspergillus* spp. However, data point scattering precluded the setting of the ECV of posaconazole and *C. glabrata*, and only tentative endpoints were listed with this species and both fluconazole and voriconazole.

For these ECVs to be clinically useful, the MIC should be determined using the same procedure. ECVs are established statistically and are based solely on multilaboratory, large numbers of MICs without any clinical information (e.g., the PK/PD parameters and the clinical response). Categorization of an isolate as WT or non-WT is not equivalent to being susceptible or resistant, respectively; that is the role of the BP. Instead, and in the absence of method-specific BPs, the ECV detects those isolates that may possess an acquired resistance factor, a factor that may or may not play a role in the actual response clinically. However, it could serve as an alert of the potential resistance of the isolate.

MATERIALS AND METHODS

Isolates. The nonserial isolates evaluated were recovered from blood cultures (candidemia patients) and other sterile and nonsterile sites (bronchoalveolar lavage fluid, sputum, respiratory related infections, or as colonizers [mostly the *Aspergillus* species isolates]). MICs from both studies and other sources were mostly determined by the bioMérieux Etest method by following the manufacturer's instructions at the following 23 medical centers: VCU Medical Center, Richmond, VA, USA; CHU de Nîmes, Montpellier, France; Unit of Mycology, Department of Microbiological Surveillance and Research, Statens Serum Institut, Copenhagen, Denmark; CHU Henri Mondor, APHP, Paris, France; CHU de Montpellier, Montpellier, France; Hospital Universitario La Fe, Valencia, Spain; Service de Parasitologie-Mycologie, CHU Toulouse, Université Paul Sabatier, Toulouse, France; Laboratoire de Parasitologie-Mycologie, Département de Microbiologie, Européen Georges Pompidou, Paris, France; Laboratoire de Parasitologie Mycologie, Rouen cedex, France; Service de Parasitologie-Mycologie, CHU Bordeaux, France; Hospices Civils de Lyon, Institut des Agents Infectieux, Parasitologie-Mycologie Médicale, Université Lyon 1, Lyon, France; AP-HP, Groupe Hospitalier Pitié-Salpêtrière, Service de Parasitologie-Mycologie, F-75013, Paris, France; The University of Alberta, Edmonton, AB, Canada; Laboratorio de Micología y Diagnóstico Molecular, Fac. Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Santa Fe, Argentina; Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico; National Institute for Communicable Diseases, Johannesburg, South Africa; Univ Rennes, CHU, Inserm, Irset (Institut de Recherche en Santé, Environnement et Travail), UMRS 1085, F-35000 Rennes, France; Servicio de Microbiología Clínica y Enfermedades Infecciosas-VIH, Hospital General Universitario Gregorio Marañon, and Instituto de Investigación Sanitaria Gregorio Marañón, Madrid, Spain; Université de Paris, UMR 261 MERIT, IRD, F 75006, Paris, France; the Innsbruck Medical University, Innsbruck, Austria; the Hospital Universitario Central de Asturias, Fundación para la Investigación Biosanitaria del Principado de Asturias, Oviedo, Asturias, Spain; Institute of Hygiene and Medical Microbiology, Medical University of Innsbruck,

Austria; and Servicio de Micologia, Laboratorio de Microbiologia, Hospital Britanico, Buenos Aires, Argentina.

Candida isolates were identified at each medical center by phenotypic features, Vitek 2 YST system, or API ID32C and, mostly, matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (37-39). The *Aspergillus* isolates were identified by microscopic morphology, internal transcribed sequences (ITS), and MALDI-TOF calmodulin sequencing (37, 40). The *Candida* mutants were identified by ITS (37). Given that molecular identification was not performed for all the isolates included in the present study, they were listed as members of the complexes of *C. glabrata*, *C. parapsilosis*, or *Aspergillus* spp. However, *C. albicans*, *C. glabrata*, and *C. krusei* submitted as having mutations were screened in the participant laboratories using published protocols (28, 30, 31). In addition, we received a set of Etest caspofungin MICs for 70 isolates of *A. fumigatus* SS, as discussed above.

The following triazole Etest MICs for the 66 mutants (*Erg11* gene mutations) were collected: 6 *C. albicans*, 5 *C. glabrata*, 2 each *C. guilliermondii* and *C. krusei*, 45 *C. parapsilosis*, and 6 *C. tropicalis*. These isolates have been evaluated for the presence of either intrinsic or acquired azole resistance mechanisms in each of the four laboratories providing them (28, 30, 31).

The Etest data were collated from the participant laboratories for three different sets of isolates: (i) triazole MICs for 17,242 *C. albicans*, 244 *C. dubliniensis*, 5,129 *C. glabrata* SC, 275 *C. guilliermondii*, 1,133 *C. krusei*, 519 *C. lusitaniae*, 2,947 *C. parapsilosis* SC, and 2,214 *C. tropicalis* isolates originating from 3 to 23 different laboratories; (ii) amphotericin B MICs from 7 to 13 laboratories for 190 *C. guilliermondii*; 481 *C. kefyr*; 330 *C. lusitaniae*; and (iii) 3,212 caspofungin MICs for *A. fumigatus* SC, 232 *A. flavus* SC, 181 *A. niger* SC, and 267 *A. terreus* SC.

At least one of the following quality control (QC) isolates/reference isolates was used by the participating laboratories: *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 or *Paecilomyces variotii* ATCC MYA-3630 or the reference isolate *A. fumigatus* ATCC MYA-3626, *A. fumigatus* ATCC 204305, or *A. flavus* ATCC MYA-204304 (14). MIC data were only submitted for the study when the MIC ranges for the QC isolates were within the established range.

Antifungal susceptibility testing. The >95% bioMérieux Etest MICs were obtained at each center according to the manufacturer's instructions as follows. Inoculum concentrations were measured to the turbidity of a 0.5 McFarland standard and 1.5% RPMI 1640 agar with 2% glucose; the Etest strip gradient concentrations ranged from 0.002 to >128 μg/ml (antifungal dependent) (13). Etest MICs were obtained by visual observation after 24 to 48 h of incubation for *Candida* spp. and 72 h (growth dependent) for *Aspergillus* spp. The MIC was the lowest drug concentration at which the pointed end of the inhibition ellipse intercepted the scale on the antifungal strip; small colonies inside the ellipse were ignored for triazoles and echinocandins but not for amphotericin B (13).

Definitions. The definitions of the ECV, WT, and non-WT MIC isolates can be found in various publications and reference documents as described above (8, 10, 25). In brief, categorization as non-WT indicates lower susceptibility to the agent being evaluated compared to the WT isolate (no phenotypic resistance). One of the important steps during the ECV definition is the analysis of modal variability of the laboratories entering the pool; it is acceptable to allow for the inherent variability of the test (usually within one doubling dilution). In addition, the ECV should encompass ≥97% of isolates. It is also important that ECVs should be based on the method used to provide the MIC/MEC results due to the potential and confirmed difference of ECVs from two susceptibility methods. ECVs also were calculated by the criteria established by the CLSI (8, 10, 25).

Data analysis. Etest MICs were converted to the reference doubling dilution MIC scales, and distributions of each species/triazole/amphotericin B/caspofungin that originated from each center were listed in Microsoft Excel spreadsheets. We pooled distributions mostly from more than four laboratories and for >200 isolates. The following distributions were eliminated (8): (i) when the particular mode for a distribution was more than 1 to 2 dilutions from the most common mode, (ii) when the mode was not clearly defined, or (iii) where the distribution was truncated within the putative wild-type population. On three occasions one of the laboratories included in the pooled distribution provided ≥50% of the MIC data. Thus, these data point distributions were weighted equally to reduce bias in the estimate (*Aspergillus* non-*fumigatus*). Due to the excess variability problems, we could not define ECVs for *C. glabrata* and posaconazole. Following the elimination of abnormal distributions, the resulting pooled distributions were used to calculate ECVs by the iterative statistical method (25). This was implemented in a Microsoft Excel macro-enabled workbook as ECOFFinder v2.1 (https://www.clsi.org/meetings/microbiology/ecoffinder/). The modeling method approaches the putative wild-type population from the low end, thereby eliminating or minimizing the impact of overlapping non-wild-type populations that might be present in the complete aggregated distribution (25). Each resulting Etest ECV captured ≥95% or ≥97.5% of the modeled WT population. Although no decision has been reached regarding the preferred ECV when the two values are different, e.g., 95 versus 97.5%, we have listed both ECVs in Tables 2 and 6.

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