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ABSTRACT Method-dependent Etest epidemiological cutoff values (ECVs) are not available for susceptibility testing of either *Candida* or *Aspergillus* species with amphotericin B or echinocandins. In addition, reference caspofungin MICs for *Candida* spp. are unreliable. *Candida* and *Aspergillus* species wild-type (WT) Etest MIC distributions (microorganisms in a species-drug combination with no detectable phenotypic resistance) were established for 4,341 *Candida albicans*, 113 *C. dubliniensis*, 1,683 *C. glabrata* species complex (SC), 709 *C. krusei*, 767 *C. parapsilosis* SC, 796 *C. tropicalis*, 1,637 *Aspergillus fumigatus* SC, 238 *A. flavus* SC, 321 *A. niger* SC, and 247 *A. terreus* SC isolates. Etest MICs from 15 laboratories (in Argentina, Europe, Mexico, South Africa, and the United States) were pooled to establish Etest ECVs. Anidulafungin, caspofungin, micafungin, and amphotericin B ECVs (in micrograms per milliliter) encompassing ≥97.5% of the statistically modeled population were 0.016, 0.5, 0.03, and 1 for *C. albicans*; 0.03, 1, 0.03, and 2 for *C. glabrata* SC; 0.06, 1, 0.25, and 4 for *C. krusei*; 8, 4, 2, and 2 for *C. parapsilosis* SC; and 0.03, 1, 0.12, and 2 for *C. tropicalis*. The amphotericin B ECV was 0.25 μg/ml for *C. dubliniensis* and 2, 8, 2, and 16 μg/ml for the complexes of *A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*, respectively. While anidulafungin Etest ECVs classified 92% of the *Candida fks* mutants evaluated as non-WT, the performance was lower for caspofungin (75%) and micafungin (84%) cutoffs. Finally, although anidulafungin (as an echinocandin surrogate susceptibility

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marker) and amphotericin B ECVs should identify *Candida* and *Aspergillus* isolates with reduced susceptibility to these agents using the Etest, these ECVs will not categorize a fungal isolate as susceptible or resistant, as breakpoints do.

KEYWORDS ECVs, Etest ECVs, Etest MICs Candida, Etest MICs Aspergillus, WT isolates, amphotericin B resistance, antifungal resistance, echinocandin resistance, non-WT, susceptibility marker

The incidence and prevalence of invasive infections caused by *Candida*, *Aspergillus*, and other fungal pathogens continue to increase, especially among immunocompromised patients and those with serious underlying diseases; their attributable mortality rates can be as high as 47% depending on the patient population and age (1–4). Three echinocandins (anidulafungin, caspofungin, and micafungin) have been licensed for intravenous treatment and prevention of invasive *Candida* infections (including candidemia) (5). The echinocandins and amphotericin B also serve as alternative choices to the triazoles as salvage, empirical, prophylactic, and/or adjunctive therapies for invasive aspergillosis (6).

The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have established standard guidelines for testing the susceptibilities of *Candida* spp. to echinocandins, including species-specific breakpoints (BPs) and epidemiological cutoff endpoints (ECVs) for MIC interpretation (7–13). While both organizations also have developed standard methodologies for testing amphotericin B against *Candida* and *Aspergillus* spp. (7, 12, 14), CLSI has set ECVs and EUCAST BPs for various *Candida* and *Aspergillus* species (10, 11, 15–17). Species-specific BPs predict the likelihood of clinical success of treatment. In the absence of BPs, the method-dependent and species-specific ECVs should identify non-WT isolates with reduced susceptibility to the agent under evaluation due to acquired mutational or other resistance mechanisms (11, 18). In the case of *Candida* isolates, echinocandin-resistant and/or non-WT isolates often harbor amino acid substitutions in Fks1p (and/or Fks2p in *C. glabrata*) genes and have been associated with breakthrough candidiasis (19) or treatment failure (20). Similar information is not available for amphotericin B, given that polyene resistance mechanisms in *Aspergillus*, *Candida*, or any fungal species are not yet as clearly determined/understood and resistance is unusual.

Significant interlaboratory variability in caspofungin modal MICs (wide modal ranges) precludes routine testing or reporting of reference caspofungin MICs for *Candida* spp. derived by both CLSI and EUCAST methodologies (21); unreliable MICs could lead to incorrectly interpreting susceptibility results. However, caspofungin modal variability does not pose a problem when using the Sensititre YeastOne (SYO) colorimetric method for susceptibility testing of *Candida* spp. and echinocandins (22), which prompted the establishment of method-dependent echinocandin SYO ECVs for *Candida* spp. (23). Another commercial and frequently used method for antifungal susceptibility testing is the gradient agar diffusion-based Etest assay (bioMérieux, Marcy l'Etoile, France) (24). Both commercial methods recommend the use of CLSI interpretive criteria of MIC results for *Candida* spp. (23, 25). ECVs could be useful in the surveillance of *in vitro* resistance and to distinguish between phenotypic wild-type (WT; no detectable phenotypic resistance) and non-WT isolates that are less likely to respond to contemporary therapy. This is important when limited clinical data have precluded the development of BPs for most fungal species.

The ECV is the highest MIC/MEC (minimum effective concentration) in a distribution of the WT population and is established by using reliable MIC/MEC distributions from multiple laboratories. Therefore, we have pooled Etest MIC data from multiple laboratories to define method-dependent Etest ECVs for species of *Candida* and *Aspergillus* versus echinocandins and amphotericin B. These data are representative of the susceptibility of these species to the agents evaluated by the Etest. Although caspofungin MEC distributions for *Aspergillus* spp. were also collected, significant heterogeneity in the respective modal MECs, similar to that described for reference caspofungin and

Candida spp. MIC distributions (21), precluded the establishment of Etest ECVs for species of *Aspergillus* and caspofungin; these results will be presented separately.

In the present study, we have (i) aggregated echinocandin and amphotericin B MICs generated using the Etest agar diffusion method originating from 3 to 10 laboratories for six *Candida* species (*Candida albicans*, *C. dubliniensis*, *C. glabrata* species complex [SC], *C. krusei* [*Issatchenkia orientalis*], *C. parapsilosis* SC, and *C. tropicalis*) and four *Aspergillus* species complexes (*A. fumigatus*, *A. flavus*, *A. niger*, and *A. terreus*) in order to define the WT MIC distributions of amphotericin B, anidulafungin, caspofungin, and micafungin; and (ii) have proposed method-dependent Etest ECVs for each of these amphotericin B- and echinocandin-species combinations for which the number of Etest MIC values was at least 100. Additional MIC distributions comprising MIC values from 25 to 62 isolates of less prevalent species (*C. guilliermondii* [*Meyerozyma guilliermondii*], *C. lusitaniae* [*Clavispora lusitaniae*], and *C. kefyr* [*Kluyveromyces marxianus*]) were also documented. Echinocandin and amphotericin B MICs for 25 to 4,341 isolates (species and agent dependent) were pooled by using data collected in 3 to 10 (of 15) independent laboratories (in Argentina, Europe, Mexico, South Africa, and the United States).

Given that most of the isolates included in the study were not assessed for mechanisms of resistance, we evaluated the application of our ECVs using Etest MIC data for individual well-characterized non-WT (isolates harboring mechanisms of resistance or *fks1* and *fks2* gene mutations) (19, 26–33) and WT (no phenotypic resistance or *fks* gene mutations) isolates, in the same manner that CLSI and SYO echinocandin ECVs were evaluated in previous studies (9, 23).

RESULTS AND DISCUSSION

BPs are unique and reliable predictors of clinical response to therapy for the isolate/agent being evaluated. Both CLSI and EUCAST have established BPs for the more prevalent *Candida* spp. and some antifungal agents (8, 17). Although BPs are not available for most fungal species-agent combinations, CLSI ECVs have been established for more fungal species (both prevalent and less common) based solely on reference *in vitro* data (10, 11, 18). The Etest method has been extensively evaluated for testing both yeasts and molds and the antifungal agents included in the present study (34–41) to estimate essential and categorical agreement with reference methods or the potential Etest's superior value as a predictor of antifungal resistance. Given that the Etest relies on CLSI BPs for clinical interpretation of results, method-dependent Etest ECVs could be useful in the clinical setting. In this study, we have proposed Etest ECVs for three echinocandins and amphotericin B and the six more prevalent species of *Candida*, as well as amphotericin B ECVs for four complexes of *Aspergillus*, according to the criteria set forth by the CLSI (10, 11) (see Table 3). These Etest ECVs could aid the clinician and laboratory personnel in identifying echinocandin and amphotericin B potential resistance (non-WT isolates) instead of relying on CLSI interpretive criteria (BPs) (25); they also could be most useful for surveillance or epidemiological purposes, especially for amphotericin B resistance, which is rare.

Although Etest MIC data were received from 15 laboratories, not all laboratories provided distributions for all species and agents evaluated. There were also exclusions due to either aberrant (mode at the lowest concentration tested) or bimodal distributions or when MICs for the quality control (QC) isolates were outside the recommended range (10, 11) The aggregated Etest MIC distributions of the three echinocandins for the common and less prevalent *Candida* spp. included are listed in Table 1. Table 2 presents pooled Etest MIC data of amphotericin B for species and SC of *Candida* and *Aspergillus*. The Etest echinocandin and amphotericin B modal MICs from individual participant laboratories were similar for each species-agent combination evaluated (within one 2-fold dilution). However, that was not the case among caspofungin MECs for *Aspergillus* spp., as mentioned above (data not included in Tables 1 and 2). In contrast, modal MEC variability was not observed in the study that established CLSI ECVs for caspofungin and *Aspergillus* spp. (42). As expected, caspofungin MIC modes for *Candida* spp.

TABLE 1 Echinocandin pooled MIC distributions for species of *Candida*[c]

Agent and species[a]	No. of laboratories	No. of isolates tested	No. of isolates with MIC (μg/ml) of[b]:												
			0.002	0.004	0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	≥8
Anidulafungin															
C. albicans	6	988	132	288	**362**	107	31	31	18	9	5		4	1	
C. glabrata SC	5	461	1	15	58	**281**	69	17	9	6	2	1	1		1
C. krusei	6	198			7	51	**91**	27	10	**7**	3	1			1
C. parapsilosis SC	4	309				2	2	3	4	13	20	84	**107**	47	27
C. tropicalis	5	197	3	18	46	**97**	20	7	3	2				1	
Caspofungin															
C. albicans	9	2,537			9	26	151	549	**876**	596	263	52	4	2	9
C. glabrata SC	10	1,148			3	10	22	77	287	**446**	237	51	10	4	1
C. krusei	5	189				1		3	2	45	**115**	19	3		1
C. parapsilosis SC	6	631			2		2	6	56	79	**209**	199	53	14	11
C. tropicalis	8	796				16	23	131	176	**266**	148	30	6		
C. guilliermondii	3	25					1	4	**8**	5	4	2	1		
C. kefyr	3	27						1	6	6	**12**	2			
Micafungin															
C. albicans	6	878	19	74	278	**333**	95	34	18	6	8	7	1	5	
C. glabrata SC	6	381	6	72	**139**	121	18	13	7	4	1				
C. krusei	4	139				4	10	41	**63**	15	4	2			
C. parapsilosis SC	6	336					4	6	15	18	**168**	81	35	8	1
C. tropicalis	5	140		4	16	31	**48**	25	10	4	1	1			

[a]SC, species complex.
[b]The highest number in each row (showing the most frequently obtained MIC or the mode) is in boldface.
[c]Data are from between 3 and 10 laboratories and were determined by the commercial Etest agar diffusion method.

were higher than those of anidulafungin or micafungin; the exception was the anidulafungin mode for *C. parapsilosis* SC (2 versus 0.5 μg/ml) (Tables 1 and 3). Overall, the modes of anidulafungin were substantially lower than those of the other two echinocandins (*Candida* spp.) and amphotericin B (both species of *Candida* and *Aspergillus*), with the exception noted above for the combination of anidulafungin and *C. parapsilosis* SC. The highest amphotericin B modes were for *C. krusei* (1 μg/ml), *A. flavus* SC (2 μg/ml), and *A. terreus* SC (4 μg/ml) (Tables 2 and 3). In general, similar Etest data have been previously published (34–41), but as observed in prior ECV studies, our MIC ranges tended to be wider, especially for *Candida* spp. versus anidulafungin and micafungin.

Table 3 also summarizes the proposed Etest ECVs for the species and agents with sufficient data to fulfill the current criteria (>100 Etest MICs of each echinocandin or amphotericin B from ≥3 laboratories) for establishing method-dependent ECVs (10, 11).

TABLE 2 Amphotericin B pooled MIC distributions for species of *Candida* and *Aspergillus*[c]

Species[a]	No. of laboratories	No. of isolates tested	No. of isolates with MIC (μg/ml) of[b]:										
			≤0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	>8
C. albicans	9	4,341	58	112	269	559	1,079	**1,435**	701	94	24	10	
C. glabrata SC	8	1,683	3	5	16	39	134	347	**785**	287	55	11	1
C. dubliniensis	4	113	5	10	12	**45**	27	9	5				
C. krusei	6	709	1	8	11	18	26	95	190	**250**	91	15	4
C. parapsilosis SC	7	767	2	5	15	69	90	224	**236**	90	24	12	
C. tropicalis	9	645	1	12	14	36	68	161	**213**	106	24	9	1
C. guilliermondii	4	54		1	6	15	19	8	**3**	2			
C. kefyr	3	54					9	11	23	9	2		
C. lusitaniae	4	62			2	17	17	**22**	12	2			
A. fumigatus SC	10	1,637		1	6	11	102	260	**726**	446	61	13	11
A. flavus SC	6	238					3	12	18	63	**79**	42	21
A. niger SC	7	321		1	2	2	24	92	**135**	48	15	1	1
A. terreus SC	6	247					2	3	9	36	69	**85**	43

[a]SC, species complex.
[b]The highest number in each row (showing the most frequently obtained MIC or the mode) is in boldface.
[c]Data are from between 3 and 10 laboratories and were determined by the commercial Etest agar diffusion method.

TABLE 3 Method-dependent Etest ECVs of caspofungin, anidulafungin, micafungin, and amphotericin B for species of *Candida* and *Aspergillus*[f]

Agent and species[a]	No. of isolates tested	MIC (μg/ml)		Method-dependent ECVs[e] (μg/ml)			
		Range	Mode[b]	Etest[c] (≥97.5%)	SYO (97.5%)	CLSI (≥97.5%)	EUCAST
Anidulafungin							
C. albicans	988	0.002–4	0.008	0.016	0.12	0.12	0.03
C. glabrata SC	461	0.002–8	0.016	0.03	0.12	0.12	0.06
C. krusei	189	0.008–8	0.03	0.06	0.25	0.25	0.06
C. parapsilosis SC	309	0.016–32	2	8/8[d]	4	8	4
C. tropicalis	197	0.002–4	0.016	0.03	0.5	0.06	0.06
Caspofungin							
C. albicans	2,537	0.08–32	0.12	0.5	0.25	NA	NA
C. glabrata SC	1,148	0.08–32	0.25	1	0.25	NA	NA
C. krusei	189	0.016–8	0.5	1	1	NA	NA
C. parapsilosis SC	631	0.08–32	0.5	4	2	NA	NA
C. tropicalis	796	0.016–2	0.25	1/1[d]	0.25	NA	NA
Micafungin							
C. albicans	878	0.002–4	0.16	0.03	0.06	0.03	0.015
C. glabrata SC	381	0.002–0.5	0.008	0.03	0.03	0.03	0.03
C. krusei	139	0.016–1	0.12	0.25	0.25	0.25	0.25
C. parapsilosis SC	336	0.03–16	0.5	2	4	4	2
C. tropicalis	140	0.004–1	0.03	0.12/0.12[d]	006	0.06	0.06
Amphotericin B							
C. albicans	4,341	0.008–4	0.25	1	NA	2	1
C. glabrata SC	1,683	0.016–16	0.5	2	NA	2	1
C. krusei	709	0.008–32	1	4	NA	2	1
C. parapsilosis SC	767	0.016–4	0.5	2	NA	2	1
C. tropicalis	645	0.008–32	0.5	2	NA	2	1
C. dubliniensis	113	0.008–0.5	0.06	0.25/0.5[d]	NA	NA	NA
Amphotericin B							
A. fumigatus SC	1,637	0.016–32	0.5	2	NA	2	1
A. flavus SC	238	0.12–32	2	8	NA	4	4
A. niger SC	321	0.016–8	0.5	2	NA	2	1
A. terreus SC	247	0.12–32.	4	16	NA	4	4

[a]SC, species complex.
[b]Most frequent MIC in the distribution. NA, not available.
[c]Calculated Etest ECVs comprising ≥97.5% of the statistically modeled population; SYO, CLSI, and EUCAST ECVs based on MICs determined by the SYO (23), CLSI M27A-3 (9), and EUCAST (denominated as ECOFFS; www.eucast.org [12]) broth dilution methods in previous studies.
[d]ECVs obtained before and after normalization of data for the analysis, when the distribution of one of the laboratories provided ≥50 of the data (10, 11).
[e]NA, not available.
[f]Data are based on MICs from between 3 and 10 laboratories determined by the commercial Etest agar diffusion method.

Method-dependent caspofungin Etest ECVs for *Candida* spp. and amphotericin B ECVs for both *Candida* and *Aspergillus* were consistently either the same or higher (one to two dilutions) than those for the SYO or CLSI methods (9, 11, 23). In contrast, available ECVs of the other two echinocandins were either mostly the same (micafungin) or up to three dilutions lower (anidulafungin). Overall, EUCAST ECVs were more comparable to Etest cutoffs (17). These discrepancies among the ECVs for those different methodologies emphasize the importance of using the interpretation criteria of the method used to obtain the susceptibility testing result. The high ECVs for certain species (values above expected and achievable serum levels) indicate the resistant nature of these species to the agent evaluated and suggest that they could be unresponsive to therapy with that agent. After all, a categorization of an isolate as WT does not necessarily signify that it is susceptible or treatable.

Table 4 depicts the ability of the proposed echinocandin Etest ECVs to identify non-WT *Candida* isolates (with *fks1* [all species] or *fks2* [*C. glabrata* only] gene mutations). The total of 140 molecularly defined echinocandin mutants included 60 *C. albicans*, 51 *C. glabrata*, 18 *C. krusei*, and 11 *C. tropicalis* isolates (19, 26–33). However, anidulafungin and micafungin Etest MICs were only available for 116 and 102 strains,

TABLE 4 Application of method-dependent Etest ECVs of caspofungin, anidulafungin, and micafungin to identify non-WT *Candida* strains harboring *fks1* and *fks2* gene mutations[a]

Agent and species	No. of mutants identified/no. tested[b] (%)	No. of mutants			ECV[c] (μg/ml)
		Below ECV	At ECV	Above ECV	
Caspofungin					
C. albicans	51/60 (85)	7	2	51	0.5
C. glabrata	34/51 (67)	12	5	34	1
C. krusei	11/18 (61)	6	1	11	1
C. tropicalis	9/11 (ND)	0	2	9	1
Total	105/140 (75)				
Anidulafungin					
C. albicans	48/55 (87)	1	6	48	0.016
C. glabrata	36/37(97)	0	1	36	0.03
C. krusei	14/15 (93)	0	1	14	0.06
C. tropicalis	9/9 (ND)	0	0	9	0.03
Total	107/116 (92)				
Micafungin					
C. albicans	43/50 (86)	5	2	43	0.03
C. glabrata	27/31 (87)	1	3	27	0.03
C. krusei	8/12 (67)	4	0	8	0.25
C. tropicalis	8/9 (ND)	0	1	8	0.12
Total	86/102 (84)				

[a]Mutation data are from references 19 and 26–33.
[b]Gene mutations present in the isolates misclassified as WT among the isolates for which anidulafungin data were available were the following: *C. albicans*, FKS1 L644L/stop F641L S645F (2 isolates) and R647I S645P (2 isolates); *C. glabrata*, FKS2 K1323E; *C. krusei*, FKS1 T657I L660I. ND, not determined due to small values.
[c]Calculated Etest ECVs comprising ≥97.5% of the statistically modeled population.

respectively. Despite that, Etest anidulafungin ECVs of 0.016 μg/ml for *C. albicans*, 0.03 μg/ml for *C. glabrata* SC, 0.06 μg/ml for *C. krusei*, and 0.03 μg/ml for *C. tropicalis* correctly classified 107 of those 116 (92%) mutants as non-WT (MICs were greater than the ECVs). Performance of Etest ECVs of anidulafungin was similar to those for SYO (89%) and CLSI (92%) ECVs for the same *Candida* spp. (9, 23). On the other hand, using the cutoffs listed in Tables 3 and 4 for caspofungin and micafungin, the Etest correctly identified a much lower number of mutants (75 and 84%, respectively) than CLSI (100% with micafungin cutoffs), SYO (91% and 94 with caspofungin and micafungin cutoffs, respectively) (9, 23), and our Etest anidulafungin ECVs (Table 4). The Etest dip effect phenomenon that appears to increase caspofungin MICs for common *Candida* spp. (especially for values of ≤0.25 μg/ml) may explain the lower performance of our Etest caspofungin ECVs in identifying *Candida* mutants (43). However, it does not explain the similarly poor performance of the micafungin Etest ECVs. The main problem was with *C. glabrata* SC (Table 4). The presence of *FKS* gene mutations in isolates is not always associated with *in vitro* or phenotypic resistance; we observed that among the mutants for which anidulafungin Etest MICs were available, the same gene mutation was present in four isolates of *C. albicans*, FKS1 S645F (2 isolates) and S645P (2 isolates) (Table 4). Testing caspofungin or micafungin by the Etest is probably not the best approach until more Etest data for *Candida* mutants, especially for micafungin, are available. Given the increasing incidence of echinocandin resistance among *Candida* spp. (*C. glabrata* in particular), evaluating the susceptibilities of *Candida* isolates to anidulafungin using the Etest can serve as a more specific surrogate marker for emerging echinocandin resistance, as has been suggested for CLSI and EUCAST methodologies and caspofungin (44).

As mentioned above, similar genetic information is not available for amphotericin B and species of either *Candida* or *Aspergillus*. Although in the 1990s the Etest was reported to be superior to the CLSI method in its ability to detect amphotericin B resistance (based on *in vivo*, not genotypic, data) in *Candida* spp. (45), that study only included one isolate each of *C. albicans* and *C. tropicalis* (Etest MICs, 32 μg/ml).

Amphotericin B MICs for the four resistant *C. lusitaniae* isolates evaluated in that study were 1 to ≥4 μg/ml; it is interesting that 1 μg/ml was the upper value for the pooled amphotericin B MIC distribution for this species from the four laboratories that provided those data in our study (Table 2). Most of the method-dependent ECVs of amphotericin B in Table 3, especially for *Aspergillus* spp., are the same as or above the expected serum concentrations by contemporary dosages, as well as above the conventional and EUCAST susceptible BPs of 1 μg/ml. ECVs that could be beyond achievable serum concentrations have previously been defined (for *Fusarium* spp., among others) as well as in the present study (echinocandins and *C. parapsilosis*) (Table 3) (9, 11, 23). They indicate the intrinsic resistant nature of those species to available agents and that they could be unresponsive to treatment with contemporary therapy. For example, most amphotericin B MICs for *A. terreus* isolates are high in this and other studies (mode, 4 μg/ml; Table 3), and dissemination and poor outcomes with the different amphotericin B formulations are usually observed (6, 15, 16). However, there are other factors that can have or have more influence on the patient's response to therapy. The scarcity of clinical data showing a relationship between low and high MICs/MECs and response to therapy precludes more conclusive statements and the establishment of CLSI amphotericin B BPs versus any fungal pathogen and by the EUCAST for a few species. When the clinical BP is available for the method used for susceptibility testing for the isolate and agent evaluated, that BP is the endpoint that must be used. It is noteworthy that most available anidulafungin and micafungin ECVs by the different methods (Table 3) are below the CLSI susceptible BPs (8); the exceptions again are the ECVs for *C. parapsilosis*.

In conclusion, we propose method-dependent and species-specific Etest ECVs of anidulafungin for *C. albicans*, *C. glabrata* SC, *C. krusei*, *C. parapsilosis* SC, and *C. tropicalis* of 0.016 μg/ml, 0.03 μg/ml, 0.06 μg/ml, 8 μg/ml, and 0.03 μg/ml, respectively. The anidulafungin ECVs and the Etest method were able to identify 92% of the mutants as non-WT isolates, which is the role of the ECV. In the meantime, given that the overall performance of method-dependent Etest ECVs of caspofungin and micafungin was much lower (75 and 84%, respectively), the anidulafungin Etest ECVs may serve as a surrogate marker to screen for echinocandin resistance in *Candida* spp. by this method until further information is gathered for micafungin data for mutants. We also have proposed species-specific and method-dependent Etest ECVs of amphotericin B of 1 μg/ml for *C. albicans*, 0.25 μg/ml for *C. dubliniensis*, 2 μg/ml for *C. glabrata* SC, 4 μg/ml for *C. krusei*, 2 μg/ml for *C. parapsilosis* SC, and 2 μg/ml for *C. tropicalis*, as well as ECVs of 2 μg/ml for the complexes of *A. fumigatus* and *A. niger*, 8 μg/ml for *A. flavus*, and 16 μg/ml for *A. terreus*. Due to the lack of BPs for the interpretation of Etest MICs for these agent-species-SC combinations, the ECVs proposed in our study for *Candida* spp. (amphotericin B and anidulafungin) and *Aspergillus* spp. (amphotericin B) should aid laboratory personnel as well as physicians in identifying those non-WT isolates having presumptive acquired echinocandin or amphotericin B resistance mechanisms. However, the ECV does not categorize an isolate as susceptible or resistant to the agent evaluated as breakpoints do, because they do not account for the pharmacology of the antifungal agent or the findings from clinical outcome studies.

MATERIALS AND METHODS

Isolates. The isolates evaluated were recovered from blood cultures, patients with candidemia (~90%), deep infections, other sterile sites, and other sites (bronchoalveolar lavage fluid, sputum, and other respiratory related infections [most *Aspergillus* species isolates]) (>90%). Antifungal susceptibility testing for each nonserial unique isolate was performed by the Etest gradient agar diffusion-based method by following the manufacturer's instructions (24) at the following medical centers: VCU Medical Center, Richmond, VA, USA; Unit for Mycology, Statens Serum Institut, Copenhagen, Denmark; Grupo Infección Grave, Instituto Investigación Sanitaria La Fe, Valencia, Spain; Instituto Nacional de Enfermedades Infecciosas Dr. C. G. Malbrán, Buenos Aires, Argentina; Hôpital Européen Georges Pompidou, Paris, France; Hospital Universitario La Paz, Madrid, Spain; Universidad Autonóma de Nuevo León, Monterrey, Nuevo León, México; National Institute for Communicable Diseases, Johannesburg, South Africa; Hospital Valme, Seville, Spain; Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria; Facultad de Medicina, Universidad de Córdoba, H. U. Reina Sofía, Córdoba, Spain; Hospital Universitario Puerta del Mar, Cádiz, Spain; Servicio de Microbiología, Hospital

Universitario Central de Asturias, Asturias, Spain; and University of Pittsburgh Medical Center, Pittsburgh, PA, USA.

Although MICs were received from 15 laboratories (coded 1 to 15), some laboratories only provided data for either *Candida* or *Aspergillus* species. Therefore, the data used for the analyses of each species-agent combination originated from 3 to 10 laboratories (Tables 1 and 2). Isolates of *Candida* spp. were identified at each laboratory using conventional methodologies (e.g., morphology on cornmeal-Tween 80 agar, growth at 45°C, API 32C AUX yeast identification system [bioMérieux], Vitek yeast biochemical card [bioMérieux], or mass spectrometry since 2010 [matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Bruker Corporation, Billerica, MA, USA]) or both phenotypic and/or molecular identification as needed (e.g., when the phenotypic profiles were inconclusive and were from the laboratories that reported data for the less prevalent species [internal transcribed spacer sequencing for *Candida* spp.]) (46). Isolates of *Aspergillus* spp. were also identified by conventional (both macro- and micromorphology and thermotolerance at 50°C) and molecular (mass spectrometry and internal transcribed spacer sequencing) methodologies to the species complex level (47).

We pooled the collected Etest MIC data of each agent for 4,341 *C. albicans*, 113 *C. dubliniensis*, 1,683 *C. glabrata* SC (*C. glabrata sensu stricto*, *C. nivariensis*, and *C. bracarensis*), 709 *C. krusei*, 767 *C. parapsilosis* SC (*C. parapsilosis sensu stricto*, *C. metapsilosis*, and *C. orthopsilosis*), and 796 *C. tropicalis* isolates, as well as for other less prevalent species from three to four laboratories (*C. guilliermondii*, *C. lusitaniae*, and *C. kefyr*) (Tables 1 and 2). We also pooled data for the four most prevalent complexes of *Aspergillus* (1,637 *A. fumigatus*, 238 *A. flavus*, 321 *A. niger*, and 247 *A. terreus* isolates) originating from 6 to 10 independent laboratories. One or both QC isolates (*C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258) were used during testing by the participant laboratories (22).

Etest MICs for 140 isolates tested for the presence of *fks1* or *fks2* gene mutations (non-WT strains) (19, 26–33), and 37 WT (no *fks* gene mutations) isolates also were used to assess the ability of proposed Etest ECVs of anidulafungin, caspofungin, and micafungin to identify non-WT *Candida* isolates. Etest MICs for these isolates were obtained in six of the participant laboratories. To our knowledge, similar information is not available for amphotericin B and either *Candida* or *Aspergillus* species.

Antifungal susceptibility testing. MICs were obtained at each center by following the manufacturer's instructions for the Etest agar diffusion-based method (final inoculum concentrations to the turbidity of a 0.5 McFarland standard, solidified [1.5% agar] RPMI 1640 medium with 2% glucose, and Etest gradient concentration strips that ranged from 0.002 to 32 μg/ml) (24). Etest MICs were obtained by visual observation after 24 h of incubation (or when sufficient growth permitted MIC determination), and 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 echinocandins but not for amphotericin B (24).

Caspofungin discrepant MICs (lower) for the QC strains were occasionally reported for both QC *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 isolates, as well as for the QC strain *C. parapsilosis* ATCC 22019 with amphotericin B. However, MIC data were not included in the pooled distributions used for the calculation of ECVs unless the data for QC reference strains (*C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019) were within the MIC limits listed in the informational table provided by the manufacturer (25).

Definitions. The definition of the ECV, as well as of the two populations (WT and non-WT MIC populations/isolates), have been widely provided in detail elsewhere and described above (10, 11, 18). A non-WT organism shows reduced susceptibility to the agent being evaluated compared to the WT (no phenotypic resistance) population. In addition to MIC distributions, the ECV calculation took into account each laboratory distribution mode, the inherent variability of the test (usually within one doubling dilution), and that the ECV should encompass ≥97% of isolates. Most published ECVs are based on reference MIC distributions, and ECVs based on other methods could be different. We used the same criteria and requirements for establishing CLSI and SYO method-dependent ECVs for proposed Etest cutoffs in the present study.

Data analysis. Data were analyzed as previously described (10, 11, 18), but Etest MICs were converted to the reference double dilution MIC scales (Tables 1 and 2). Etest MIC distributions of each species-SC-echinocandin-amphotericin B combination received from each center were listed in Microsoft Excel spreadsheets. Pooled distributions from ≥3 laboratories and ≥100 isolates were screened for (i) abnormal distributions (truncated distributions that had the mode at the lowest or highest concentration tested and/or that were bimodal inside the presumptive wild-type distribution), (ii) presumptive wild-type modes of more than one 2-fold dilution from the most common mode, and (iii) the percentage of isolates provided by each laboratory for each species-agent combination. For the few occasions when one of the laboratories included in the pooled distribution provided ≥50% of the MIC data, such MIC distributions were weighted (or normalized) to reduce bias in the estimate (Table 3). However, decisions have not been made regarding the preferred ECV when the two values are different, e.g., the ECV for *C. dubliniensis* and amphotericin B and others (Table 3). Following the elimination of abnormal distributions, the resulting qualifying pooled distributions were used to calculate ECVs by the iterative statistical method (10, 11, 18). Each resulting Etest ECV was the MIC that captured ≥97.5% of the modeled WT population.

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