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Abstract

Objectives: To determine the Etest-based epidemiological cut-off values (ECVs) for antifungal agents against the most frequent yeast and Aspergillus fumigatus species isolated in 12 French hospitals. Methods: For each antifungal agent, the Etest MICs in yeast and A. fumigatus isolates from 12 French laboratories were retrospectively collected from 2004 to 2018. The ECVs were then calculated using the iterative statistical method with a 97.5% cut-off.

Results: Forty-eight Etest ECVs were determined for amphotericin B, caspofungin, micafungin, anidula- fungin, fluconazole, voriconazole, posaconazole and itraconazole, after pooling and analysing the MICs of 9654 Candida albicans, 2939 Candida glabrata SC, 1458 Candida parapsilosis SC, 1148 Candida tropicalis, 575 Candida krusei, 518 Candida kefyr, 241 Candida lusitaniae, 131 Candida guilliermondii and 1526 Aspergillus fumigatus species complex isolates. These ECVs were 100% concordant (identical or within one two-fold dilution) with the previously reported Etest-based ECVs (when available), and they were concordant in 76.1% of cases with the Clinical and Laboratory Standards Institute ECVs and in 81.6% of cases with the European Committee on Antimicrobial Susceptibility Testing ECVs.

Conclusions: On the basis of these and other previous results, we recommend the determination of method-dependent ECVs. Etest ECVs should not be used instead of breakpoints, but may be useful to identify non-wild-type isolates with potential resistance to antifungal agents, and to indicate that an isolate may not respond as expected to the standard treatment.

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Introduction

The prevalence of invasive infections due to fungal pathogens continues to increase in immunocompromised individuals. In France, candidiasis represents the primary cause (49%) of invasive fungal diseases, of which 56% are due to Candida albicans, 18.6% to Candida glabrata, 11.5% to Candida parapsilosis, and 9.3% to Candida tropicalis. Aspergillosis is in third position (16% of all invasive fungal diseases) [1]. To manage these invasive fungal diseases, three classes of antifungal agents (polyenes, echinocandins and azoles) are recommended as initial or salvage therapy [2,3]. The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) have established standard procedures for testing the susceptibility of fungi to anti- fungal agents, and have proposed species-specific breakpoints for interpreting the MICs of some antifungal agents against the most prevalent Candida spp. [4e6]. Species-specific breakpoints predict the likelihood of clinical response to antimicrobial therapy. How- ever, there are many pathogeneantifungal agent combinations for which clinical breakpoints are not available, because of insufficient data to correlate the clinical outcomes with the in vitro results. When only MIC data are available, epidemiological cut-off values (ECVs or ECOFF) should be considered. ECVs are defined as the highest susceptibility end point of the MIC distribution for the wild- type (WT) population. A MIC value higher than the ECV suggests that the isolate may have developed resistance to that agent, so an alternative compound should be considered [7,8]. ECVs are dependent on the in vitro sensitivity testing method used to generate the MIC values. Although ECVs for several organismeantifungal agent combinations have been published [9e19], few data are available on ECVs based on the MIC distribu- tions obtained with commercial methods. As the gradient agar
diffusion-based Etest assay (bioMe'rieux, Marcy l'Etoile, France) is
often used in the clinical routine [10,11,13], we collected retro- spectively the MICs for yeast and Aspergillus fumigatus species complex (SC) isolates obtained with the Etest method at 12 French mycological laboratories to determine the ECVs of the main agents used in the clinical practice, in order to harmonize the interpreta- tion of the Etest MIC results.

Material and methods

MIC data collection

The MICs for different antifungal agents tested on cultured pa- tient isolates with the Etest from 2004 to 2018 were collected retrospectively from the laboratories of 12 French university hos- pitals (CHU de Nîmes, CHU de Rennes, CHU de Toulouse, CHU de Bordeaux, Hospices Civils de Lyon, CHU de Rouen, CHU de Mont- pellier, CHU de Limoges, APHP Hôpital universitaire Pitié-Sal- pêtrière, APHP Hôpital Bichat-Claude Bernard, APHP Hôpital Européen Georges Pompidou and APHP Hôpital Henri Mondor).

Species identification and antifungal susceptibility testing

The tested isolates were from routine specimen cultures (blood cultures, sterile sites and other sites, such as bronchoalveolar lavage, sputum) that required antifungal susceptibility testing for therapeutic management. Fungi were identified using different methods: phenotypic features in chromogenic medium, microscopic morphology, VITEK®2 YST system (bioMe'rieux), API® ID32C (bioMe'rieux) or mass spectrometry (Bruker Microflex LT™ system, BrukerDaltonik, Bremen, Germany, or VITEK® MS, bioMe'rieux). The Etest-based MIC values for the following species could be collected: C. albicans, C. glabrata SC, C. parapsilosis SC, C. tropicalis, Candida krusei, Candida kefyr, Candida lusitaniae, Candida guillier- mondii and A. fumigatus SC. Each laboratory determined the isolate susceptibility to different antifungal drugs (amphotericin B, anidulafungin,
caspofungin, micafungin, 5-fluorocytosine, fluconazole, itracona- zole, posaconazole and voriconazole) using the Etest gradient diffusion method, according to the manufacturer's instructions [20]. Etest MICs were determined by visual observation at 48 h of growth for yeasts, and between 16 and 72 h, depending on their growth, for A. fumigatus SC. During the study period, all contrib- uting laboratories tested the quality control reference strains. The MICs obtained for these strains were all within the expected reference ranges. All Etest MIC readings were performed by quali- fied operators. In 2018, in parallel with data collection, to test the inter-operator variability among centres, each reader determined the MICs based on visual inspection of 16 photographs of Etest assays.

No significant difference between the determined MICs was observed.

ECV determination

To ensure that robust and comparable data were included for the ECV estimation, the following basic requirements and criteria needed to be fulfilled [9]. (i) MICs were converted into the standard two-fold dilution scale based on dilution factor values that are powers of 2 (at the upper dilution). All data were collected in the form of number of isolates with different MIC values on the power of 2 scale. The MIC mode was the MIC value with the highest number of representative isolates. (ii) If the MIC mode of a distri- bution was at the lowest tested concentration, or if the distribution appeared truncated before or after the MIC mode, that distribution was excluded. (iii) Distributions with aberrant MIC modes were excluded: for example, when the MIC mode of the distribution was two-fold or more than two-fold dilutions above or below the most frequent WT mode. (iv) MIC data were not pooled if there was no obvious common mode among the range of distributions (for example bimodal distributions). (v) If one of the participating lab- oratories contributed >50% of the values to the pooled data, the MIC data were normalized to reduce this bias in the estimate. (vi) MIC data were pooled only if generated by at least three independent laboratories. (vii) For each speciesantifungal agent combination, a minimum of 100 MIC values were required after data pooling.

The ECVs from pooled data were estimated using the iterative statistical method (ISM) described by Turnidge et al. [7] and implemented in a MICROSOFT EXCEL® ECOFFINDER workbook (https://www.clsi.org/meetings/microbiology/ecoffinder/). This method selects the log normal distribution for subsequent modelling. From the real MIC distribution (power of 2 scale), the ISM attempts to fit iteratively the observed WT counts in a log normal distribution and creates a range of possible ECVs. This fitted log-normal distribution is a probability distribution of the WT population. Each resulting
Etest ECV corresponded to the MIC that captured 97.5% of the
modeled WT population, and represented the probability for an isolate to be a WT isolate if its MIC was lower or equal to the ECV value.

Ethics

This study included only data from fungal isolates. The opinion of an Institutional Review Board was not required because human participants were not involved.

Results

Analysis of the data allowed determination of 48 ECVs for nine yeast and one mould species (total number of included isolates: 9654 C. albicans, 2939 C. glabrata SC, 1458 C. parapsilosis SC, 1148 C. tropicalis, 575 C. krusei, 518 C. kefyr, 241 C. lusitaniae, 131 C. guilliermondii and 1526 A. fumigatus SC). Depending on the species and antifungal agent, MIC data from 3 to 12 laboratories were pooled. The MIC distributions for each speciesmolecule combination are presented in Table 1.

The Etest-based ECVs of amphotericin B and echinocandins for Candida spp. are presented in Table 2. ECVs were determined for the five main species (C. albicans, C. glabrata, C. parapsilosis SC,
C. tropicalis and C. krusei) using MIC data on 117 to 6062 isolates, according to the speciesantifungal agent combination. Enough data (>100 isolates) were collected to determine new ECVs for less prevalent species, such as C. kefyr, C. guilliermondii and C. lusitaniae.

The MICs determined for Candida spp. and azoles (Table 3) allowed the calculation of 15 ECVs. It was not possible to estimate the Etest-based ECVs for C. glabrata and azoles because of the aberrant MIC distribution observed with the Etest method (double peak). Moreover, the ECV for the C. kruseifluconazole combination was not determined because of its innate resistance to this drug.

To calculate the ECVs for A. fumigatus SC (Table 4), data on 361 to 1027 isolates were used, depending on the speciesantifungal agent combination. Two new ECVs were determined for echino- candins. The ECVs for A. fumigatus SC and amphotericin B and azoles were similar to those previously reported.

Overall, the ECV results were identical to (17/32 ECVs) or within one two-fold dilution of (15/32 ECVs) the previously reported Etest- based ECVs (Tables 2e4). They were comparable to the CLSI ECVs in 76.1% (similar or within one two-fold dilution: 35/46 ECVs) and absolutely identical in 39.1% of cases (18/46 ECVs). They were comparable to the EUCAST ECVs in 81.2% of cases (similar or within one two-fold dilution: 26/32 ECVs) and strictly identical in 28% (9/ 32 ECVs).

Table 1
Pooled MIC distributions of Candida species and Aspergillus fumigatus SC

Species	Antifungal drug	Tested isolates (n)	From number of laboratories	No. of isolates with MIC (mg/L) of[a]:																	
				0.001	0.002	0.004	0.008	0.016	0.032	0.064	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	>256
Candida albicans	Amphotericin B	6062	11	6	8	0	8	18	92	297	1079	2803	1569	159	19	1	0	1	0	2	0	0	0
	Caspofungin	5783	12	0	5	14	125	615	1572	1891	1220	294	34	5	1	2	2	1	1	1	0	0	0
	Micafungin	3752	11	0	6	59	855	1909	822	62	13	9	9	6	1	0	0	0	1	0	0	0	0
	Anidulafungin	1593	5	3	560	674	289	38	9	7	3	6	0	0	1	2	0	1	0	0	0	0	0
	Fluconazole	9654	12	0	0	0	0	5	59	519	2501	3880	1908	488	147	48	21	20	12	7	1	10	28
	Voriconazole	6020	12	5	327	1551	2161	1272	434	141	57	36	13	6	4	0	0	0	8	5	0	0	0
	Posaconazole	1005	6	0	5	13	151	428	288	72	25	8	7	6	1	1	0	0	0	0	0	0	0
Candida glabrata SC	Amphotericin B	2272	11	0	0	0	0	4	11	31	93	288	995	712	113	16	2	4	0	3	0	0	0
	Caspofungin	2292	11	0	0	3	0	16	44	312	1121	688	66	19	4	3	4	1	1	6	0	0	4
	Micafungin	1494	11	0	0	2	98	988	359	7	5	6	10	3	9	3	3	0	1	0	0	0	0
	Anidulafungin	513	5	0	0	8	67	353	61	8	4	4	0	3	1	2	1	1	0	0	0	0	0
	Fluconazole	2939	11	0	0	0	0	1	0	2	5	9	20	28	97	204	375	722	557	213	91	100	515
	Voriconazole	2304	11	0	4	8	13	16	57	120	326	409	423	266	145	117	95	72	61	172	0	0	0
	Posaconazole	455	7	0	0	0	1	3	2	5	14	24	49	56	63	46	35	14	58	85	0	0	0
Candida parapsilosis SC	Amphotericin B	981	10	0	1	0	1	5	17	28	53	155	390	271	48	9	1	0	0	2	0	0	0
	Caspofungin	1091	10	0	0	0	1	0	2	8	28	203	395	304	119	22	4	1	0	4	0	0	0
	Micafungin	839	11	0	0	2	1	2	1	0	4	24	102	388	234	73	5	3	0	0	0	0	0
	Anidulafungin	241	3	0	0	0	0	0	0	1	1	4	16	60	80	43	12	3	4	17	0	0	0
	Fluconazole	1458	12	0	0	0	0	4	8	22	111	337	445	288	124	40	24	19	11	5	0	3	17
	Voriconazole	1150	12	0	31	111	264	285	236	118	47	18	11	8	8	9	1	0	0	3	0	0	0
Candida tropicalis	Amphotericin B	787	10	0	0	0	1	1	3	29	49	146	295	222	36	5	0	0	0	0	0	0	0
	Caspofungin	787	10	0	1	1	2	18	59	208	300	163	23	6	3	2	0	0	0	1	0	0	0
	Micafungin	504	11	0	0	0	1	61	338	96	4	1	0	0	3	0	0	0	0	0	0	0	0
	Anidulafungin	213	5	0	2	7	50	123	24	4	2	0	0	0	1	0	0	0	0	0	0	0	0
	Fluconazole	1148	12	0	0	0	0	1	1	3	15	82	301	409	229	52	22	9	5	4	1	1	13
	Voriconazole	886	12	0	2	5	16	106	211	308	165	31	16	8	3	1	2	2	3	7	0	0	0
	Posaconazole	152	4	0	1	0	7	32	45	34	21	9	2	1	0	0	0	0	0	0	0	0	0
Candida krusei	Amphotericin B	534	9	0	0	0	1	1	4	6	18	40	144	207	96	15	1	0	1	0	0	0	0
	Caspofungin	565	10	0	0	0	0	0	1	3	16	139	303	81	18	4	0	0	0	0	0	0	0
	Micafungin	259	5	0	1	0	1	1	3	7	106	137	2	1	0	0	0	0	0	0	0	0	0
	Anidulafungin	117	3	0	2	0	0	27	43	37	5	3	0	0	0	0	0	0	0	0	0	0	0
	Fluconazole	414	11	0	0	0	0	0	0	0	0	0	3	1	1	0	1	21	65	81	23	47	171
	Voriconazole	575	10	0	0	0	0	2	5	20	79	183	212	49	14	7	2	1	0	1	0	0	0
Candida kefyr	Amphotericin B	353	9	0	0	1	0	2	8	26	45	90	161	16	4	0	0	0	0	0	0	0	0
	Caspofungin	418	8	0	0	0	0	8	49	113	212	33	3	0	0	0	0	0	0	0	0	0	0
	Micafungin	236	7	0	0	0	0	2	30	126	73	5	0	0	0	0	0	0	0	0	0	0	0
	Fluconazole	518	11	0	0	0	0	1	4	39	141	197	114	15	4	0	1	0	0	0	0	2	0
	Voriconazole	357	9	0	20	63	141	95	29	4	2	2	1	0	0	0	0	0	0	0	0	0	0
Candida lusitaniae	Amphotericin B	170	9	0	0	0	1	0	7	7	28	70	47	5	5	0	0	0	0	0	0	0	0
	Caspofungin	149	7	0	0	0	0	3	5	14	39	59	24	4	1	0	0	0	0	0	0	0	0
	Fluconazole	241	10	0	0	0	0	2	9	21	44	83	54	13	2	2	3	2	3	1	0	0	2
	Voriconazole	168	8	1	20	39	67	23	3	3	5	3	3	1	0	0	0	0	0	0	0	0	0
Candida guilliermondii	Amphotericin B	131	6	0	0	0	1	12	14	28	25	34	15	1	1	0	0	0	0	0	0	0	0
	Caspofungin	120	5	0	0	0	0	0	0	2	10	34	41	23	4	2	1	0	0	3	0	0	0
	Fluconazole	111	5	0	0	0	0	0	0	0	0	1	10	26	31	9	10	4	5	3	2	0	10
	Voriconazole	115	5	0	0	0	2	25	26	22	6	5	7	6	3	0	1	0	3	3	0	0	6
Aspergillus fumigatus SC	Amphotericin B	1027	9	0	1	0	0	1	6	9	63	225	505	178	27	7	3	1	1	0	0	0	0
	Caspofungin	806	5	0	2	12	65	193	251	191	75	16	1	0	0	0	0	0	0	0	0	0	0
	Micafungin	361	5	0	14	90	165	75	14	1	1	1	0	0	0	0	0	0	0	0	0	0	0
	Voriconazole	1526	7	0	1	1	1	1	13	50	662	622	109	29	18	10	4	2	0	3	0	0	0
	Posaconazole	961	8	0	2	2	4	20	68	367	391	75	17	3	6	1	0	1	0	4	0	0	0
	Itraconazole	989	7	0	0	0	0	1	4	10	39	190	474	199	35	4	8	6	4	15	0	0	0

[a] The MIC mode (most frequent value) for each distribution is in bold type.

Discussion

The Etest method is widely used in the clinic, and MIC inter- pretation is based on the CLSI breakpoints that are, however, available only for some speciesdantifungal drug combinations [21]. In their absence, the method-dependent ECVs allow identification of non-WT isolates that might be resistant to an antifungal agent. ECVs for some speciesdantifungal agent combinations have been established using both reference methodsdCLSI [9,12,15e19] and EUCAST [9,10,14,22,23]dand some commercial methods, such as Sensititre YeastOne [24] and Etest [10,11,13]. In this study, we collected the MIC values (obtained with the Etest) for more than 18 000 Candida spp. and A. fumigatus SC isolates and calculated the Etest-based ECVs (n ¼ 48) using the ISM [7] at the 97.5% cut-off value to exclude isolates with extreme MICs. This analysis included the five Candida species responsible for more than 90% of invasive candidiasis and all the classes of antifungal agents rec- ommended in the guidelines [2,3] (except for C. glabrata and azoles), as well as less prevalent Candida species (C. lusitaniae, C. guilliermondii, C. kefyr), and A. fumigatus SC. Currently, only three other studies used the ISM and Etest data to calculate the ECVs (n ¼ 50) of some antifungal agents for six Candida spp. and five Aspergillus spp. [10,11,13]).

Table 2
Etest epidemiological cut-off values of amphotericin B and echinocandins in Candida spp.

	Contributing laboratories/total (n)	Used isolates/ total (n)	MIC mode (mg/L)	Etest ECV (this study)[a]	Etest ECVs (Espinel-Ingroff)[b]	CLSI ECVs[c]	EUCAST ECVs[d]	CLSI BPs S≤/R≥[e]	EUCAST BPs S≤/R>[f]
Amphotericin B									
Candida albicans	11/12	6062/6249	0.25	1	1	2	1	d	1
Candida glabrata SC	11/12	2272/2446	0.5	2	2	2	1	d	1
Candida parapsilosis SC	10/11	981/1137	0.5	2	2	2	1	d	1
Candida tropicalis	10/12	787/866	0.5	2	2	2	1	d	1
Candida krusei	9/11	534/603	1	4	4	2	1	d	1
Candida kefyr	9/11	353/380	0.5	2	d	d	d	d	d
Candida lusitaniae	9/11	170/189	0.25	1	d	2	d	d	d
Candida guilliermondii	6/11	131/164	0.25	1	d	2	d	d	d
Anidulafungin									
Candida albicans	5/5	1593/1593	0.004	0.008	0.016	0.12	0.03	0.25/1	0.03/0.03
Candida glabrata SC	5/5	513/513	0.016	0.03	0.03	0.12	0.06	0.12/0.5	0.06/0.06
Candida parapsilosis SC	3/4	241/259	2	8*	8*	8	4	2/8	0.002/4
Candida tropicalis	5/5	213/213	0.016	0.03	0.03	0.12	0.06	0.25/1	0.06/0.06
Candida krusei	3/3	117/117	0.03	0.12*	0.06	0.25	0.06	0.25/1	0.06/0.06
Micafungin									
Candida albicans	11/11	3752/3752	0.016	0.03	0.03	0.03	0.016	0.25/1	0.016/0.016
Candida glabrata SC	11/11	1494/1494	0.016	0.03	0.03	0.03	0.03	0.06/0.25	0.03/0.03
Candida parapsilosis SC	11/11	839/839	1	4	2	4	2	2/8	0.002/2
Candida tropicalis	11/11	504/504	0.03	0.06	0.12	0.06	0.06	0.25/1	d
Candida krusei	5/11	259/402	0.25	0.5	0.25	0.25	0.25	0.25/1	d
Candida kefyr	7/11	236/254	0.06	0.25	d	0.12	d	d	d
Caspofungin									
Candida albicans	12/12	5783/5783	0.06	0.25	0.5	0.12	d	0.25/1	d
Candida glabrata SC	11/12	2292/2303	0.12	0.5	1	0.12	d	0.12/0.5	d
Candida parapsilosis SC	10/12	1091/1109	0.5	2	4	1	d	2/8	d
Candida tropicalis	10/12	787/798	0.12	0.5	1	0.12	d	0.25/1	d
Candida krusei	10/12	565/568	0.5	1	1	0.25	d	0.25/1	d
Candida kefyr	8/10	418/446	0.12	0.25	d	0.03	d	d	d
Candida lusitaniae	7/10	149/175	0.25	1	d	1	d	d	d
Candida guilliermondii	5/9	120/152	0.5	2	d	2	d	2/8	d

Abbreviations: BP, breakpoint; ECV, epidemiological cut-off value; MIC mode, most frequent MIC in the distribution; R, resistant; S, susceptible; SC, species complex.
*ECV obtained after data normalization (>50% of all data were from one laboratory).
[a] ECVs calculated for the modelled population (≥97.5%).
[b] ECVs determined by Espinel-Ingroff et al. with the Etest method (≥97.5%) [13].
[c] ECVs determined using the CLSI method in previous studies [9,15e17,19].
[d] ECVs determined using the EUCAST method [9,14,23].
[e] CLSI breakpoints [15,21].
[f] EUCAST breakpoints [6].

Table 3
Etest epidemiological cut-off values for azoles and Candida spp.

	Contributing laboratories/ total (n)	Used isolates/ total (n)	MIC mode (mg/L)	EtestECVs (this study)[a]	EtestECVs (Espinel-Ingroff)[b1]	Etest ECVs (EUCAST determination)[b2]	CLSI ECVs[c]	EUCAST ECVs[d]	CLSI BPs S≤/R≥[e]	EUCAST BPs S≤/R>[f]
Fluconazole										
Candida albicans	12/12	9654/9654	0.25	1	d	1	0.5	1	2/8	2/4
Candida glabrata SC	0/11	0/2939	d	d	64	32	8	32	SDD≤32/R ≥ 64	0.002/32
Candida parapsilosis SC	12/12	1458/1458	0.5	2	4	2	1	2	2/8	2/4
Candida tropicalis	12/12	1148/1148	1	4	4	2	1	2	2/8	2/4
Candida krusei	11/11	414/414	>256	d	d	128	32	128	d	d
Candida kefyr	11/12	518/527	0.25	1	d	d	1	d	d	d
Candida lusitaniae	10/10	241/241	0.25	1	d	d	1	16	d	d
Candida guilliermondii	5/10	111/186	2	4	d	d	8	16	d	d
Voriconazole										
Candida albicans	12/12	6020/6020	0.008	0.03	0.03	0.12	0.03	0.12	0.12/1	0.06/0.25
Candida glabrata SC	0/11	0/2304	d	d	2	1	0.25	1	d	d
Candida parapsilosis SC	12/12	1150/1150	0.016	0.12	0.25	0.12	0.03	0.12	0.12/1	0.12/0.25
Candida tropicalis	12/12	886/886	0.06	0.25	0.5	0.12	0.06	0.12	0.12/1	0.12/0.25
Candida krusei	10/12	575/673	0.5	1	2	1	0.5	1	0.5/2	d
Candida kefyr	9/11	357/374	0.008	0.03	d	d	0.016	d	d	d
Candida lusitaniae	8/11	168/189	0.008	0.03	d	0.06	0.03	0.06	d	d
Candida guilliermondii	5/10	115/163	0.03	0.12	d	0.25	0.12	0.25	d	d
Posaconazole										
Candida albicans	6/7	1005/1014	0.016	0.06	0.12	d	0.06	0.06	d	0.06/0.06
Candida glabrata SC	0/7	0/455	d	d	d	d	2	1	d	d
Candida tropicalis	4/6	152/159	0.03	0.25*	0.12	d	0.12	0.06	d	0.06/0.06

Abbreviations: BP, breakpoint; ECV, epidemiological cut-off value; MIC mode, most frequent MIC in the distribution; R, resistant; S, susceptible; SC, species complex.
*ECV obtained after data normalization (>50% of all data were from one laboratory).
[a] ECVs calculated for the modelled population (≥97.5%).
[b1] ECVs determined by Espinel-Ingroff et al. with the Etest method (≥97.5%) [11].
[b2] Etest-based ECVs determined by the EUCAST organization [23].
[c] ECVs determined with the CLSI method in previous studies [9,11,12,17].
[d] ECVs determined with the EUCAST method [5,9,14,23].
[e] CLSI breakpoints [15,21].
[f] EUCAST breakpoints [6].

For the Candida spp.eamphotericin B combinations, we deter- mined eight ECVs, of which three were new and five displayed the same values as those reported by Espinel-Ingroff et al. [13].

For the Candida spp.-echinocandin combinations, our Etest ECVs were identical (6/15 ECVs) or within one two-fold dilution (9/15 ECVs) compared with the previously published values. These non- significant discrepancies could be explained by the higher number of isolates analysed in our study (e.g. 5783 C. albicans isolates for caspofungin and 504 C. tropicalis isolates for micafungin versus 2537 and 140 isolates, respectively, in [13]). Espinel-Ingroff et al. showed that the Etest ECVs for anidulafungin identified 92% of FKS mutants as non-WT isolates (versus 75% and 84% with the ECVs for caspofungin and micafungin, respectively) [13]. The authors concluded that the Etest ECVs for anidulafungin could represent a surrogate marker for echinocandin resistance screening in Candida spp., as also suggested by Pfaller et al. with the CLSI method [25]. In our study, the ECVs for caspofungin were most often lower (one two-fold dilution) than those reported by Espinel-Ingroff et al., which raised the question of selecting a robust surrogate marker for echinocandin resistance screening. To determine the most useful echinocandin ECV to identify non-WT isolates, it might be neces- sary to test isolates with characterized mutations.

For the Candida spp.eazole combinations, among the Etest- based ECVs previously published [11], two were similar and six were within one two-fold dilution. Other Etest ECVs are available for azoles in the EUCAST website (https://mic.eucast.org/Eucast2/) [23], but they were determined using a small number of strains, sometimes <100 observations for some speciesantifungal agent combinations. There was no significant difference (i.e. more than one two-fold dilution) between our ECVs and the EUCAST ECVs, but for the C. albicansevoriconazole combination (ECV ¼ 0.03 mg/L in our study and 0.12 mg/L by EUCAST). However, our ECV is identical to the one reported by Espinel-Ingroff et al., suggesting that this is a more robust value. We could not determine the Etest ECVs for C. glabrata SC and azoles because of the aberrant MIC distribution (double peak). Indeed, there was a significant number of strains with high MICs (>256 mg/L) due to the appearance of 'macro-col- onies' in the inhibition ellipse when MICs were read at 48 h. Some authors have found lower rates of agreement for C. glabrata and azoles when comparing the Etest and the CLSI method (higher MICs with the Etest) [26,27]. The incubation time seems to influence the results. Indeed, a better agreement between these methods is observed

at 24 h of growth and reading. In their latest study to determine the ECVs for C. glabrata SC and azoles, Espinel-Ingroff et al. read the Etest MICs between 24 h and 48 h, depending on the growth [11]. This may explain why they did not observe double peaks and could, therefore, estimate the ECVs.

In our study, we could determine the Etest ECVs for antifungal agents used as first-line and also salvage therapy of the most prevalent species of invasive aspergillosis (A. fumigatus SC). In contrast to our study, Espinel-Ingroff et al. observed a significant heterogeneity in the minimum effective concentration mode for Aspergillus spp. and caspofungin, and could not determine the Etest ECVs [13]. The new identification methods (especially matrix- assisted laser desorption/ionization time-of-flight) that can also identify cryptic species will allow determination of the ECVs for species within a complex. This is important because the suscepti- bility to an antifungal drug is not the same for all species within a complexdfor example, Aspergillus lentulus shows higher MICs to amphotericin B) [28].

Table 4
Etest epidemiological cut-off values for the Aspergillus fumigatus species complex

	Contributing laboratories/total (n)	Used isolates/ total (n)	MIC mode (mg/L)	EtestECVs (this study)[a]	Etest ECVs (Espinel-Ingroff)[b]	CLSI ECVs[c]	EUCAST ECVs[d]	CLSI BPs S/R	EUCAST BPs S/R[e]
Voriconazole	7/10	1526/1626	0.12	0.5	0.5	1	1	d	1/2
Posaconazole	8/10	961/1035	0.12	0.25	0.25	0.25	0.25	d	0.125/0.25
Itraconazole	7/8	989/1064	0.5	2	2	1	1	d	1/2
Amphotericin B	9/10	1027/1039	0.5	2	2	2	1	d	1/2
Caspofungin	5/9	806/892	0.03	0.12*	d	0.5	d	d	d
Micafungin	5/8	361/372	0.008	0.016*	d	d	d	d	d

Abbreviations: BP, breakpoint; ECV, epidemiological cut-off value; MIC mode, most frequent MIC in the distribution; R, resistant; S, susceptible; SC, species complex
*ECV obtained after data normalization (>50% of data were from one laboratory).
[a] ECVs calculated for the modelled population (97.5%).
[b] ECVs based on the Etest method determined by Espinel-Ingroff et al. (97.5%) [10,11,13].
[c] ECVs determined with the CLSI method in previous studies [10,13].
[d] ECVs determined with the EUCAST method [5,9,10,23].
[e] EUCAST breakpoints [6].

All the previously published ECVs were calculated by only one team. Our multi-laboratory study allowed consolidation of these previous ECV data and validation of our results. Specifically, 17/32 of our ECVs are identical to previous ones, and 15/32 show just one two-fold dilution [10,11,13], a difference that we consider not significant (our definition is more stringent than the essential agreement, which considers two log2 dilutions). These not significant differences can be explained by the higher number of laboratories and isolates included in our study for the ECV calculations. However, ideally only one ECV should be proposed for each speciesantifungal combination. For that, our data and the previous data should be combined and analysed again to reach a consensus.

Comparison of our Etest ECVs with the ECVs obtained with the reference methods indicated that they were identical to or within one two-fold dilution of the CLSI ECVs in 76.1% of cases [9,12,15e17,19] and to the EUCAST ECVs in 81.2% of cases [9,10,14,22]. Discrepancies among ECVs obtained with different methods were previously reported [13,24], emphasizing the importance of using ECVs specific for the in vitro method used to test the antifungal susceptibility. Moreover, for each speciesantifungal combination, it is possible to know whether the MIC distributions obtained with the Etest are close to the distributions obtained with the CLSI and EUCAST methods. For example, for C. lusitaniae and fluconazole, the Etest MIC distributions were identical to those obtained with the CLSI but not the EUCAST method (difference of four log2 dilutions). This can be useful for using the available breakpoints.

As Etest is the most widely used commercial method in French laboratories, the goal of this study was to calculate specific ECVs for this method. Inter-method variability and comparison of the Etest method with a reference method were previously performed with good essential agreement [26,29,30]. It is now important to have data on Etest MIC distributions for MIC interpretation in routine clinical practice and for the detection of non-WT isolates. When the MIC value is higher than the ECV, the search for possible gene mutations to that antifungal agent should be recommended.

Conclusions

This study identified 48 ECVs specific for the Etest method to facilitate MIC interpretation. They should not be used instead of breakpoints, but they may be useful to identify non-WT isolates with potential resistance to antifungal agents and to suggest that an isolate may not respond as expected to the standard treatment. ECVs have a place in the surveillance and monitoring of the emergence of drug resistance. It will be interesting to prospectively collect and pool MIC data to confirm the calculated Etest ECVs and to determine other ECVs, especially for rare species. Testing isolates with acquired resistance mutations characterized by molecular methods will also provide a way to verify these ECVs.

Transparency declaration

The authors declare that there are no conflicts of interest rele- vant to this article.

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