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Review

The role of epidemiological cutoff values (ECVs/ECOFFs) in antifungal susceptibility testing and interpretation for uncommon yeasts and moulds

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ABSTRACT

The role of antimicrobial susceptibility testing is to aid in selecting the best agent for the treatment of bacterial and fungal diseases. This has been best achieved by the setting of breakpoints by Clinical Laboratory Standards Institute (CLSI) for prevalent *Candida* spp. versus anidulafungin, caspofungin, micafungin, fluconazole, and voriconazole. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) also has set breakpoints for prevalent and common *Candida* and *Aspergillus* species versus amphotericin B, itraconazole, and posaconazole. Recently, another interpretive category, the epidemiological cut off value, could aid in the early identification of strains with acquired resistance mechanisms. CLSI has postulated that epidemiological cut off values may, with due caution, aid physicians in managing mycosis by species where breakpoints are not available. This review provides (1) the criteria and statistical approach to establishing and estimating epidemiological cut off values (ECVs), (2) the role of the epidemiological cut off value in establishing breakpoints, (3) the potential role of epidemiological cut off values in clinical practice, (4) and the wide range of CLSI-based epidemiological cut off values reported in the literature as well as EUCAST and Sensititre Yeast One-ECVs. Additionally, we provide MIC/MEC (minimal inhibitory concentrations/minimum effective concentrations) ranges/modes of each pooled distribution used for epidemiological cut off value calculation. We focus on the epidemiological cut off value, the new interpretive endpoint that will identify the non-wild type strains (defined as potentially harboring resistance mechanisms). However, we emphasize that epidemiological cut off values will not categorize a fungal isolate as susceptible or resistant as breakpoints do, because the former do not account for the pharmacology of the antifungal agent or the findings from clinical outcome studies.

Utilidad clínica de los puntos de corte epidemiológicos (ECVs/ECOFFs) para interpretar los datos de la sensibilidad antifúngica de los mohos y levaduras de poca prevalencia

Palabras clave:
Resistencia antifungica
Puntos de corte
EUCAST
CLSI
ECVs/ECOFFs

RESUMEN

Las pruebas de sensibilidad a los antimicrobianos tienen como finalidad ayudar en la selección del mejor fármaco para el tratamiento de las infecciones fúngicas y bacterianas. El establecimiento de puntos de corte para la anidulafungina, la caspofungina, la micafungina, el fluconazol y el voriconazol en las especies de *Candida* más prevalentes por parte del Clinical and Laboratory Standards Institute (CLSI) permite alcanzar este objetivo. El European Committee on Antimicrobial Susceptibility Testing (EUCAST) también ha establecido puntos de corte para la anfotericina B, el itraconazol y el posaconazol en las especies más comunes de *Candida* y *Aspergillus*. En los últimos tiempos se ha propuesto una nueva categoría, la de los puntos de corte epidemiológicos, que puede ayudar a identificar de manera temprana los aislamientos que han adquirido mecanismos de resistencia. Según el CLSI

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los puntos de corte epidemiológicos podrían, con la debida cautela, ayudar a los médicos en la selección del tratamiento en aquellas micosis causadas por especies para las que no se han establecido puntos de corte. Esta revisión repasa: 1) los criterios y la aproximación estadística seguida para establecer y estimar los puntos de corte epidemiológicos, 2) el papel de los puntos de corte epidemiológicos para establecer los puntos de corte, 3) el papel de los puntos de corte epidemiológicos en la práctica clínica, y 4) el amplio rango de puntos de corte epidemiológicos que aparecen en la literatura establecidos mediante los métodos del CLSI, EUCAST o Sensititre® YeastOne®. Se muestran también los rangos de las concentraciones mínimas inhibitorias (CMI) y concentraciones mínimas efectivas (CME) utilizados para el cálculo de los puntos de corte epidemiológicos. Incidimos de manera especial sobre estos últimos por tratarse de una nueva interpretación de los CMI y los CME que permite identificar aquellos aislamientos que no son salvajes y potencialmente resistentes. No obstante, insistimos en que los puntos de corte epidemiológicos no pueden utilizarse para calificar como resistente o sensible a un determinado aislamiento, como lo hacen los puntos de corte, puesto que los puntos de corte epidemiológicos no se rigen por las características farmacológicas de los agentes antifúngicos ni por la evolución clínica de los pacientes.

Although most fungi associated with disease are considered opportunistic pathogens (in particular the yeasts), irrespective of the species, fungi cause a great deal of morbidity and mortality worldwide, especially among the increasing number of immunocompromised patients.[27,34] Such patients are at high risk for life-threatening mycoses. Despite the available antifungal agents (amphotericin B and its lipid formulations, the triazoles and the echinocandins), the mortality rates and emergence of resistance have increased, especially among patients suffering invasive infections due to *Aspergillus* and other filamentous fungi (moulds) infections; e.g., the survival rates could be below 30% depending on the infecting isolate and the immunological status of the host in invasive fusariosis.[1,33] There is therefore a strong motivation to ensure that antifungal therapy is optimized, and consequently that susceptibility testing maximizes its capacity to predict outcome.

Advances have been made in understanding the molecular mechanisms of resistance in some *Candida* spp., *Cryptococcus* spp. and *Aspergillus* spp. and the triazoles as well as in *Candida* spp. with the echinocandins. For example, the emergence of triazole resistance among patients with aspergillosis has been reported where isolates with *CYP51A* gene mutations have reduced azole affinity or increased target quantity that confers azole resistance; this problem has increased significantly in some European countries.[47] However, the genetics of other important species (e.g., *Fusarium* spp. and the Mucorales) are yet to be investigated. Therefore, there is a need to identify these resistant isolates with reduced antifungal susceptibility.

Currently the best predictors of patient outcome with antifungal therapy are the formal breakpoints (BPs) established by the standards setting organizations, the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI). However, CLSI BPs are only available for some *Candida* spp. versus two triazoles and three echinocandins,[6] while EUCAST had established BPs for the prevalent *Candida* spp. and some common *Aspergillus* spp. for three other antifungal agents (amphotericin B, itraconazole, posaconazole). BPs by definition are used to indicate those isolates that are likely to respond to (susceptible) or fail (resistant) treatment with a given antimicrobial agent administered at the approved dosing regimen for that agent (http://www.eucast.org/clinical_breakpoints/).[5] They are established using a combination of in vitro, in vivo and clinical data, including the distribution of the minimal inhibitory concentrations (MICs) and/or minimal effective concentrations (MECs), in vitro, animal model pharmacokinetics/pharmacodynamics (PK/PD), and clinical/microbiological outcome data (EUCAST SOP 1.1 Setting breakpoints for new antimicrobial agents at http://www.eucast.org/documents/sops/).[5]

One of the first steps in establishing BPs is the determination of what constitutes wild-type (WT) strains, defined as strains without any phenotypically-expressed resistance mechanism(s). Rather than being a single value, MICs/MECs of WT strains follow a log-normally distribution. The upper end of that distribution is defined as the epidemiological cutoff value (ECV or ECOFF). Estimation of the ECV has the added benefit of acting as a sensitive indicator of the emergence of strains with reduced susceptibility to a given agent. Over the last few years, ECVs have been proposed for a variety of moulds and *Cryptococcus* spp. as well as for some of those species of *Candida* for which BPs have not been established. Recently, method-dependent ECVs for the commercial colorimetric Sensititre® YeastOne® assay (SYO) for *Candida* spp. have been estimated; these values have been integrated in the tables, given that the high degree of caspofungin MIC variability for *Candida* spp. has so far prevented reproducible testing of this agent by reference methodology.[14] The CLSI has described guidelines and listed CLSI ECVs as per CLSI criteria, while the EUCAST publishes visual ECVs on their MIC distribution website when the data are of sufficient quality (http://mic.eucast.org/Eucast2/).

This review presents information regarding both interpretive categories or enpoints that encompass: (1) steps needed for statistical ECV estimation and/or establishment as per CLSI criteria, (2) the role of the ECV in establishing BPs, (3) the role of ECVs on clinical practice versus the BP's fundamental role, (4) and the wide range of CLSI ECVs reported in the literature as well as EUCAST and SYO-ECVs. Other interpretive endpoints that have been proposed as either BPs or ECVs especially for *Aspergillus fumigatus* species complex (SC) in the last few years are briefly discussed.

Epidemiological cutoff value estimation

Basic requirements or criteria

In order to ensure that clean and comparable data are included in the estimation of ECVs, there are a number of conditions/criteria that must be fulfilled. These include conditions that apply to the data generated in the contributing laboratory, as well as methods applied to accepting and rejecting particular distributions before pooling for analysis. The pooling of data for the estimation of the ECV (See Figs. 1 and 2) is built on the assumption that the WT of an individual species does not vary over time or place (e.g., anywhere in the world). The following are widely accepted criteria for generating, reviewing, excluding and pooling the data required for the estimation of ECVs; many of which were recently described by Kahlmeter:[25]

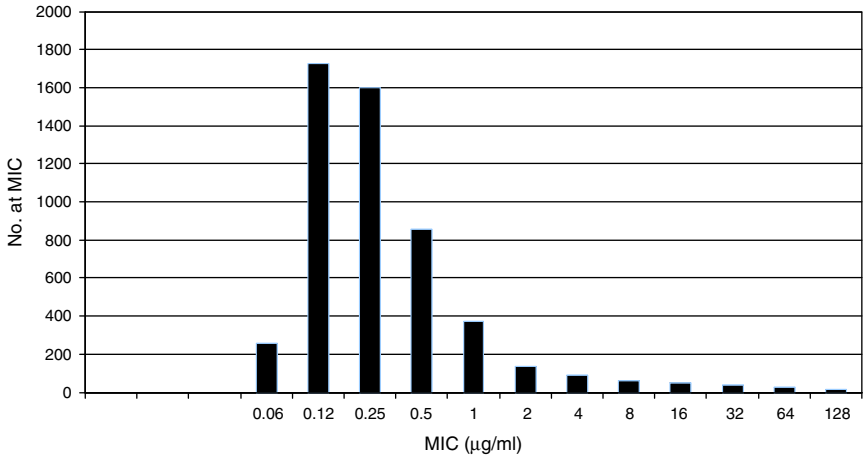

Fig. 1. Pooled fluconazole MIC distribution of *Candida albicans* from 9 laboratories. Graphical representation of the pooled nine distributions (single distributions are provided in Table 1) selected for the calculation of the CLSI ECV for *C. albicans* and fluconazole showing a shoulder on the left and similar bars between fluconazole concentrations 0.12 and 0.25 μg/ml; the mode is 0.12 μg/ml.[15]

(i) ECVs can only be set for a single species/SC; molecular identification of certain fungal groups is essential for ECV calculations since it is widely recognized that some of these common pathogens represent species complexes that cannot be identified to the species/SC level by conventional methodologies alone (e.g., members of the Mucorales, *Fusarium* spp., the complexes of *Candida glabrata*, *Candida parapsilosis* and the various *Aspergillus* spp., among others);[22]

(ii) data should only be included from unique clinical isolates; MICs/MECs of repeat isolates from a single episode of infection should be excluded;

(iii) MICs/MECs must have been determined using reference methods (M27-A3; M38-A2; http://www.eucast.org/ast_of_fungi/methodsinantifungalsusceptibilitytesting/)[3,4];

(iv) MICs/MECs must conform to the standard two-fold dilution series based on powers of 2;

(v) MIC/MEC distributions from contributing laboratories must be accompanied by MICs/MECs within established ranges for quality control (QC) isolate(s) obtained during the testing period;

(vi) MIC/MEC data must be generated by a minimum of 3 and preferably 5 independent and geographically distinct laboratories (to allow for interlaboratory variation which is quite common – see Tables 1 and 2);

(vii) there should be at least data for five isolates in each single distribution as well as a minimum of 100 MIC/MEC values after pooling (assuming it includes relatively few or no strains with acquired resistance mechanisms);

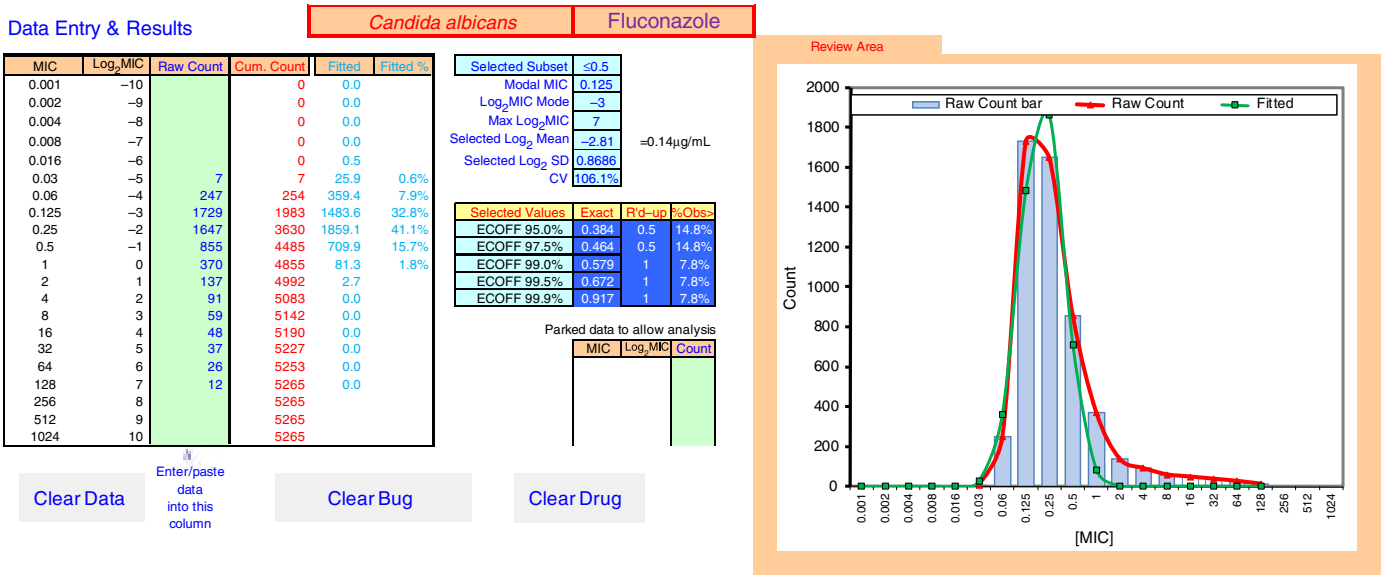

Fig. 2. ECOFFinder results for fluconazole MIC distribution of *C. albicans* from 9 laboratories. The analysis is done on a worksheet separate to that displayed and the results carried back to the Data Entry & Results sheet displayed here. The four left hand columns tabulate the pooled MIC distribution data. The "Fitted" and "Fitted %" columns show the numbers and percentage of isolates at each MIC in the estimated wild-type distribution. The central area of the screen shows, at the top, the subset of the raw data that gave the best fit and the statistical parameters that defined the log-normal distribution with the best fit. Below that are the range of ECVs that might be chosen according to the percentage of the wild-type that the user might wish to include, including the "exact" ECV, the ECV rounded up to the next two-fold dilution, and the percentage of isolates in the observed raw data that were above that rounded-up ECV. On the right is a graph displaying the raw data in both column and curve format (curve in red), and the curve (in green) of the estimated best fit wild-type population. The "Parked Data to Allow Analysis" section in the lower center is used when there is a mode in the high non-wild-type population that is greater than the mode in the wild-type population. Data at this high mode and above are moved here to retain their visibility but allow the analysis to precede, because the analysis relies on the mode of the data to run efficiently. The buttons "Clear Data", "Clear Bug" and "Clear Drug" are form controls that activate macros to clear the worksheet of some or all data entry elements for re-use of ECOFFinder.

Table 1
Single fluconazole MIC distributions of *C. albicans* from 13 laboratories.

Agent	Participant Laboratory No.													
	1	3	4	5	6	7	11	12	18	Pooled distribution	19	2	9	10
FLU MIC														
0.01														
0.03							2	5		7				
0.06	97	42	16	12	3		42	30	5	247				
0.12	**483**	67	**120**	**34**	**80**	23	**295**	**596**	31	**1729**	***5159***	***1230***		***393***
0.25	224	**115**	29	20	68	**110**	197	322	**562**	1647	2471	995	***420***	286
0.5	55	76	3	14	6	12	44	193	452	855	274	87	15	23
1	28	54	1	15	1	26	12	27	206	370	51	25		9
2	11	16	3	6	4	1	5	18	73	137	37	18	2	5
4	18	3	1	8	6	4	1	15	35	91	21	28		5
8	8	2		9		4	5	14	17	59	11	19	1	4
16	2	3		20		1	3	12	7	48	19	8	2	3
32	2	7		16		1		7	4	37	9	7	4	
64	6			18				2		26	3	5	1	2
128				1	1			5	5	12	4		2	
Total	934	385	173	173	169	182	606	1246	1397	5265	8059	2422	447	730

Pool of the 13 single fluconazole MIC distributions received for the calculation of the fluconazole ECV for *C. albicans*: On the left, the nine distributions selected for the ECV calculation (the graphical representation of the pool is seen in Figs. 1 and 2) and on the right, the four truncated distributions (modes [bolded/italics] at the lowest concentration tested) that were not included in the analysis.[15]

(viii) if the mode of a distribution is at the lowest concentration tested (e.g., truncated distributions: laboratories 2, 9, 10 and 19 in Table 1), this distribution must be excluded from pooling; distributions which appear truncated before the upper end of the presumptive WT distribution should also be excluded from pooling; truncation beyond the presumptive WT is to be expected for some fungal groups depending on intrinsic susceptibility and the prevalence of high-level resistance;

(ix) distributions with aberrant modes also must be excluded from pooling; these are defined as either having modes that are two or more two-fold dilutions above or below the most frequent WT mode of the individual distributions, or distributions where the mode is not obvious (e.g., laboratory 12 in Table 2);

(x) in situations where there is no obvious common mode amongst the range of distributions, data should not be pooled or analyzed (e.g., data shown in Table 2)[14];

(xi) if one of the contributing distributions contributes more than 50% of the values to the pooled data, consideration should be given to giving equal weighting to all contributing distributions, to minimize bias, where different ECVs can be calculated (See Tables 3, 10 and 11). However, it is fine when both values are the same after normalizing the (weighting analysis; see example, Table 3) and the ECV is acceptable, but what steps should be taken when the two values are different is under consideration.

Table 1 shows a working example of raw MIC distributions obtained from 13 laboratories around the world. It can be seen that data from 4 laboratories (numbers 2, 9, 10 and 19) were excluded from the pooling because their modes were at the lowest dilution/concentration tested.[15] All criteria were met for the remaining 9 datasets. Fig. 1 shows the histogram of the same data after pooling. The figure shows that the distribution of the presumptive WT is approximately log-normal, and that choosing an ECV based on observation alone is likely to be quite subjective, because there is obvious skewing to the right of the distribution.

By contrast, Table 2 shows an example of a situation where, and as mentioned above, there is no obvious common mode among the distributions/laboratories (criterion x); this precludes the establishment of an ECV based on the current data.[14] Indeed, the modes span 5 dilutions, suggesting that there are significant methodological issues with testing the antifungal-species combination. Such problems are rare. In addition, and as also mentioned under criterion ix, laboratory 12 shows an aberrant mode problem, with seemingly two modes at 0.12 and 0.06 μg/ml.

Table 2
Single caspofungin MIC distributions of *C. albicans* from 14 laboratories.

Agent	MIC	Participant Laboratory No.													
		4	20	3	2	18	19	1	7	11	12	9	14	6	10
Caspofungin	**0.0079**	10	3			7	92								
	0.01	**136**	**28**	***181***	1	114	1181				141				
	0.03	13	2	31	**755**	**670**	**2037**	2	6	15	***343***				38
	0.06	6	3	55	659	544	898	**39**	6	59	218				15
	0.12	8	1	69	357	49	68	24	**128**	**72**	***345***	1	20		9
	0.25	6	3	14	212	2	6	10	12	8	117	**332**	**39**	58	106
	0.5	4		1	176	5	1	7	16	11	71	108		**97**	**552**
	1			3	30	5		1	10	1	10	3	1	13	10
	2				16	2					1			1	3
	4				4							3			
	8				10										

Pool of the 14 single caspofungin MIC distributions received for the calculation of the caspofungin ECV for *C. albicans*:
The examination of modes (bolded) in each participant laboratory indicated interlaboratory variability (up to five dilutions) which precluded the calculation of the ECV for this agent and *Candida* spp; similar phenomenon was observed for other species. Distribution from laboratory 3 was truncated and distribution from laboratory 12 was bimodal (The total number of caspofungin MICs is 11,550).[14]

Table 3
Example of weighting applied to the raw data to enable ECV estimation when one distribution accounts for more than 50% of the values for isavuconazole versus *Aspergillus fumigatus* species complex. For these data, the unweighted ECV estimated at the 97.5% level by ECOFFinder was 1 μg/ml,[13] while the weighted ECV was 2 μg/ml.

MIC (μg/ml)	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Pooled distribution
Unweighted counts									
0.01									
0.03									
0.06	2		1			3			6
0.12		3	3			25			31
0.25	10	10	5	2	4	116		2	149
0.5	45	49	14	4	26	356	6	8	508
1	19	8	10	6	12	4	48	6	113
2	6	4	3	1	3		16		33
4			1		1	1	1		4
8	3				8				11
Total	***85***	***74***	***37***	***13***	***54***	***505***	***71***	***16***	***855***
% Grand total	9.9%	8.7%	4.3%	1.5%	6.3%	59.1%	8.3%	1.9%	
Weighted counts									
0.01									
0.03									
0.06	2.4		2.7			0.6			5.6
0.12		4.1	8.1			5.0			17.1
0.25	11.8	13.5	13.5	15.4	7.4	23.0		12.5	97.1
0.5	52.9	66.2	37.8	30.8	48.1	70.5	8.5	50.0	364.9
1	22.4	10.8	27.0	46.2	22.2	0.8	67.6	37.5	234.5
2	7.1	5.4	8.1	7.7	5.6		22.5		56.4
4			2.7		1.9	0.2	1.4		6.2
8	3.5				14.8				18.3
Total	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*100*	*800*
% Grand total	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	12.5%	

Table 4
Impact of resistance mechanisms on in vitro susceptibility of *C. albicans* to voriconazole and fluconazole.

Strain[a]	Resistance mechanisms		MIC (μg/ml)	
	CDR[b]	ERG11[c]	Voriconazole	Fluconazole
DSY296	Basal	WT/WT	0.007	0.25
DSY296[d]	**Increase**	**G464S/G464S**	**2**	**64**
DSY3083	Basal	G464S/G464S	0.12	4
DSY3604	Basal	G464S/WT	0.06	2
DSY3606	Increase	WT/WT	0.12	4

[a] Single strain manipulation.
[b] Level of expression of CDR1/CDR2 efflux pumps.[15,31,35,37]
[c] Mutations (G464S) in each of 2 ERG11 alleles.[31,35]
MICs of strain DSY296 increased to resistant levels: RBP of ≥1 μg/ml and ≥8 μg/ml for voriconazole and fluconazole, respectively.[6]
MICs of the other strains would be classified as non-WT but below the resistant level: voriconazole and fluconazole ECVs are 0.03 μg/ml and 0.5 μg/ml respectively.[6,15,35,37]

Phenotype versus genotype

Application of the above criteria is done in the growing knowledge that fungal taxonomy is undergoing rapid changes at present, especially since the advent of MALDI-TOF identification and whole genome sequencing.[22] What defines a species or a SC is, in many cases, in a state of flux. Another important consideration is that acquired resistance mechanisms can frequently be detected using modern molecular techniques, and can be used to validate ECVs that have been estimated from phenotypic data. From this can follow the temptation to discard a phenotypically-determined ECV in favor of an inference of acquired resistance based solely on molecular results[46]. However, mutations in genes encoding antifungal target enzymes, by far the most common mechanism of acquired resistance to antifungal agents, may not always alter the phenotype. This could be because the mutations are 'silent', e.g., do not affect the target site of the enzyme, or because the resistance gene is not expressed. Ultimately, antifungal agents attack target enzymes, not the genes encoding them, and that is the phenotype that the drug "sees".

However, there is still great utility in examining molecular mechanisms of resistance. This may lead to improvements in ECVs by estimating the sensitivity and specificity of both phenotypic and genotypic assays. In doing so, it is necessary to undertake molecular testing of all isolates at the top of the WT range so that any overlap between resistance mechanisms and the ECV can be determined.

As previously discussed, molecular mechanisms of resistance have been described mostly for *Candida* spp. and both triazoles and echinocandins as well as for *Aspergillus* spp. and the triazoles. Azole resistance has been investigated since the 1990s when relapses among AIDS patients were associated with suboptimal fluconazole doses and high or increased fluconazole MICs; azole resistance mechanisms have been reported mostly for *Candida albicans*, *C. glabrata* and *A. fumigatus*. The relationship or influence of known azole resistance mechanisms with MICs for *C. albicans* and *A. fumigatus* are depicted in Tables 4–6 and Fig. 3. In Table 4, mutations in the *ERG11* gene or the increased level of expression of the efflux pumps elevated both fluconazole and voriconazole MICs from WT to non-WT/resistant levels in *C. albicans*.[15,31,35,37] The same impact is evident on SYO-caspofungin and anidulafungin MICs for strains of *C. albicans* harboring *fks1* gene mutations (Table 5).[18] Table 6 provides the elevated triazole MICs for *A. fumigatus* isolates harboring *Cyp51* mutations and usually lower values for WT isolates as reported in multiple studies.[21,26,28–30] Results shown in Table 6 also suggest the differential effect on triazole activity/MICs by the *Cyp51* mutation in *A. fumigatus*.

Table 5
Impact of resistance mechanisms on in vitro susceptibility of *C. albicans* to caspofungin and anidulafungin.[a]

Strain[b]	Resistance mechanisms		SYO-MIC (μg/ml)	
			Caspofungin	Anidulafungin
S-100	WT	WT/WT	0.06	0.01
S-101	S645F	**G464S/G464S**	4	1
S-102	S645P	G464S/G464S	4	1
S-103	F641S	G464S/WT	8	4

[a] Data for 4 of the 81 isolates used to evaluate the usefulness of SYO ECVs to identify non-WT isolates.[18]
[b] WT (wild type) and three isolates harboring specific *fks1* mutations or non-WT.
SYO ECVs for caspofungin and anidulafungin: 0.25 μg/ml and 0.12 μg/ml, respectively.[18]

Table 6
Relationship of triazole MICs (μg/ml) for *A. fumigatus* SC and molecular mechanisms of resistance.

Cyp51 mutation	ITR	ISA	POS	VOR
WT MIC range	0.06–1	0.5–2	0.06–2	0.5–2
CLSI ECV	1	1	0.5	1
G54	>8	0.5–2	0.25–16	0.25–2
L98	>8	8–16	0.5–1	4–8
20	>8	1–8	0.25–16	0.5–4
G138,Y431, G434, G448, F46Y	2 to >8	16	0.5–16	4–16

ITR, itraconazole; ISA, isavuconazole; POS, posaconazole; VOR, voriconazole.
Adapted from refs: [21,26,28–30].

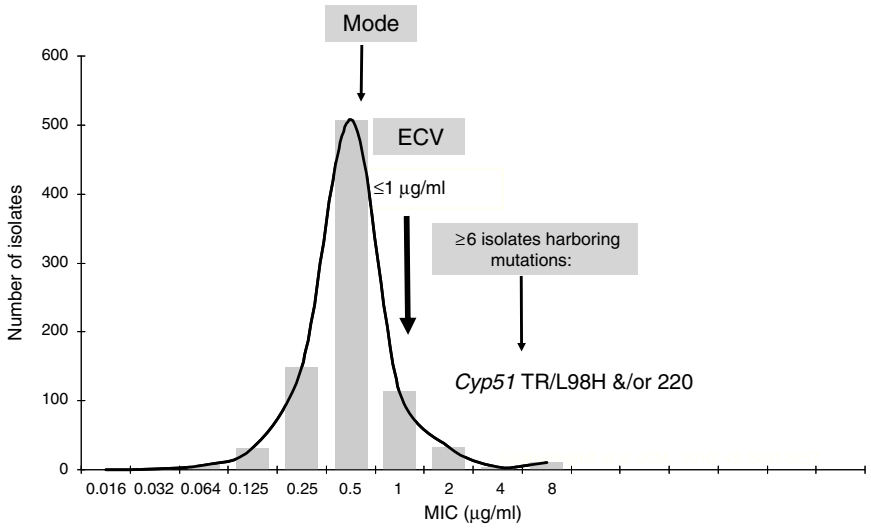

Fig. 3. Isavuconazole ECV for *A. fumigatus* complex (Note: isolates harboring gene mutations have MICs of 4 μg/ml.[13]). Graphical representation of the pooled MIC distribution from eight laboratories used for the calculation of the CLSI ECV of *A. fumigatus* SC and isavuconazole.

The application of CLSI ECVs to identify mutant isolates has provided encouraging results, e.g., echinocandin and triazole ECVs for *Candida* spp. would have identified >80% of the reported mutants evaluated in each ECV study.[15,39] That is the main role of the ECV, to identify non-WT isolates. Unfortunately, the molecular biology of resistance for many other important fungal species needs to be investigated, e.g., most non-*Aspergillus* moulds including both Mucorales and *Fusarium* spp. as well as less prevalent yeast species.

Methods for estimating ECVs from pooled data

A range of methods for estimating ECVs have been developed over the years, as summarized and added to by Cantón et al.[2] The methods can be categorized as either observational or statistical. A particular hazard with the observational methods is that they do not account for the fact that a MIC value, as it is currently determined, is actually the upper end of the MIC interval, rather than the midpoint of the interval. This can lead to overestimation of the location of the WT population as a whole. For instance, a MIC value of a single isolate recorded as 2 μg/ml actually means that the MIC for that isolate is somewhere in the range of >1 μg/ml and ≤2 μg/ml. The average MIC of a collection of isolates when the MIC/MEC is recorded as 2 μg/ml is in fact the geometric mean of 1 and 2 μg/ml, e.g., half way on a logarithmic scale between 2^0 and $2^1 = 2^{0.5}$ or ~1.414 μg/ml. Thus, the conventional histogram of MICs or MECs used for observational estimation is shifted to the right by one half of a two-fold dilution.

For the many publications referred to in this review, we have elected to use the statistical method described by Turnidge et al.,[44] which attempts to fit a log-normal distribution to the presumptive WT counts iteratively – the so-called iterative statistical method (ISM). It is implemented in a Microsoft Excel® workbook (ECOFFinder) that can be downloaded freely from the CLSI website (http://clsi.org/standards/micro/ecoffinder/). We prefer this method because it minimizes the subjectivity when compared to observational methods, and does not require the presence of a population of MICs/MECs above the WT to be effective. Other statistical methods may be equally suitable, although the ECVs generated with them may differ slightly and at most by a single two-fold dilution.[2]

The estimation performed using the ISM is implemented in such a way that it accounts for the fact that MICs are actually the values at the upper end of the two-fold interval. When the iterations have been completed, it selects the log-normal distribution with the closest fit to the observed counts, and then creates a range of possible ECVs. This range is created by selecting a desired percentage to be captured in the WT, calculating the exact ECV, and then rounding it up to the next highest two-fold dilution. The reason for this is that the fitted log-normal distribution is a probability distribution, and as such the appropriate percentage of that distribution that can be used is subject to needs. Employing a lower percentage of the modeled WT population increases the probability that all isolates less than or equal to that MIC are true WT and reduces the risk of including MICs associated with acquired resistance mechanisms. Conversely, employing a higher percentage reduces the risk of true WT isolates being misclassified as likely to possess an acquired resistance mechanism (non-WT), but increases the probability of misclassifying non-WT as WT. Ultimately, it is a trade-off, and the selected percentage should be chosen according to need. Employing a lower percentage will potentially increase the requirement to test WT isolates for acquired mechanisms that they do not possess, while employing a higher percentage will increase the risk of missing important emerging resistance mechanisms. There is currently no consensus on the most appropriate percentage, although the CLSI Antifungal Susceptibility Testing Subcommittee is using 97.5% of the modeled distribution as a starting point (CLSI documents on ECVs).

Therefore, it is important to understand that an ECV can never be guaranteed to divide WT and non-WT perfectly. Assay variation and the coarseness of the two-fold dilution scale will always leave a small chance of misclassification.

Fig. 2 shows an explanation of the application of the IDN to the pooled data from Table 1 using ECOFFinder. Here the estimated WT population has a mean MIC on the $\log_2$ scale of −2.81 μg/ml and a standard deviation of 0.87.

Other types of ECVs

The conditions for establishing ECVs described above result in what might be called "reference" ECVs. A slightly less stringent condition, that of also using data generated by susceptibility testing methods calibrated to the reference method, is employed by EUCAST.[25] Until further data are forthcoming, both the authors' papers presented in this review and the CLSI have elected to apply the more stringent condition in generating ECVs from data obtained using the reference methodology only.

With this in mind, it is possible to apply the principles of ECV setting to other data types. For instance, if multiple datasets are available from another susceptibility testing method, exactly the same rules for estimating ECVs can be applied. We calculated ECVs for data generated by the Sensititre® YeastOne® (SYO) system, widely used for antifungal susceptibility testing internationally, for some *Candida* spp. and the echinocandins.[18] We call the ECVs created in this way "method-dependent".

The ECV-setting rules (criteria i to v) can also be used for a dataset from a single laboratory. The output might be described as a "local" ECV that should only be applied to the testing performed in that laboratory. Some laboratories may choose to set a "local" ECV for quality assurance purposes and/or to maximize their chances of detecting emerging resistance.

ECVs: their role in establishing breakpoints

The generation and examination of MIC/MEC distributions is the obvious first step in the development of BPs. It is now agreed by CLSI and EUCAST that the next step is to determine the ECVs according to the criteria described above. This is important because it is considered undesirable to establish a BP that cuts into the WT distribution. The WT distribution is in fact a distribution built up from a combination of biological disparity and assay (intra- plus inter-laboratory) variation. Because assay variation is a large component of the distribution, as QC range studies demonstrate,[3,6] setting a BP that cuts into the WT will result in frequent categorization and reporting errors attributable to assay variation, but with no biological significance.

The establishment of BPs requires more steps and much more data than those for ECV calculation as follows[5,7,25,45]: (1) calculation of ECVs for each species/agent according to the steps or criteria listed above; (2) available knowledge of resistance mechanisms of the agent and their correlation with MICs/MECs and/or in vivo outcomes; (3) investigation of PK and PD parameters of the agent in animals and in human trials where possible; and (4) the correlation of MICs and clinical outcome to therapy for patients treated with the specific agent from clinical trials. The latter step is the major impediment for both moulds and less prevalent yeast species given that formal clinical trials have not been conducted or the infecting isolates are mostly non-WT. For example, the *Aspergillus* isolates from voriconazole clinical trials were WT and efficacy was associated with the site of infection.[40]

Given the comparative rarity of infections caused by moulds and other less prevalent yeast species, it may take many years or decades before sufficient reliable data will be available to establish BPs for the less common yeasts, and almost all moulds. This was the principal motivation for considering ECVs an option for reporting the results of susceptibility tests

ECVs: their potential use in clinical practice

The role of the ECV is to separate or distinguish WT from non-WT isolates.[44] Non-WT isolates harbor acquired resistance mechanisms, which most often correlate with high MICs or MECs, that are above the ECV for the species/agent being evaluated.[15,18,24,26,29–32,39] Categorization of an isolate as non-WT indicates a decreased susceptibility to the agent evaluated or potential phenotypic resistance; therefore, ECVs also have an important role in tracking the emergence of resistance. The sustained use of antifungal agents would continue to increase selection pressure for resistance. On the other hand, BPs categorize the isolate as either treatable (susceptible) or non-treatable (resistant) and therefore is the most accurate predictor of patient response to therapy.[7,25,45] BPs are not available for many antifungal agent/fungal species combinations because the data requirements for establishing BPs;[5] (EUCAST SOP 1.1 Setting breakpoints for new antimicrobial agents at http://www.eucast.org/documents/sops/) are beyond the available MIC/MEC data. In particular, the lack of clinical trials showing a relationship between low and high MICs/MECs and response to therapy, as well as knowledge regarding molecular resistance mechanisms, have hampered the establishment of BPs. However, where there are sufficient MIC/MEC data, it is possible to calculate ECVs. Because ECVs are solely based on those in vitro data, ECVs cannot be used to predict clinical response to treatment with that antifungal agent. Therefore, categorization of an isolate as WT or non-WT is not equivalent to being "susceptible" or "resistant" respectively. Instead, the role of the ECV is to identify those non-WT isolates that potentially harbor acquired resistance mechanisms. Consequently, when there is a formal BP, this is the MIC/MEC value that must be used to report "S", "SDD" or "R" to the treating clinician. In some instances the ECV is higher than anything achievable with the expected drug

exposure; for example, most high ECVs for *Fusarium* spp. indicate the resistant nature of those species to available agents.

When BPs are not available, the question arises as to whether an ECV can be used to convey information to a treating clinician. Opinions vary, but at present the CLSI are considering applying ECVs where BPs are not available and reporting to the clinician whether the isolate is WT or Non-WT as a comment, without an "S" or "R" interpretation. Should the test indicate that the isolate is Non-WT, the comment would include a statement that, if the patient is undergoing treatment with that agent, the clinician should review the patient's clinical response. How this might play out in the clinical context has yet to be determined as new clinical data are available.

Established CLSI and other ECVs

ECVs established for the CLSI reference methods

Using the rules and techniques described above, we have estimated a wide range of ECVs based on the CLSI reference methods and guidelines to define them for important species of *Aspergillus*, Mucorales and *Fusarium*, and various species of *Candida* and *Cryptococcus*. (Tables 7–11)[8–13,15–17,19] A brief description of each group is given below; Annex 1 lists the contributors to the numerous single MIC/MEC distributions used for the calculation of both CLSI and SYO ECVs.

- ***Aspergillus* species complex**. Table 7 provides CLSI ECVs for five *Aspergillus* SC and the agents more frequently used or potentially useful in the treatment of aspergillosis.[8–10,13] Data that corroborate the results in Table 7 have appeared in the literature regarding CLSI itraconazole and voriconazole ECVs of 1 μg/ml for *A. fumigatus* SC (Table 7a) as genetic examination indicated that MICs of 1 μg/ml separated *mutants* (with *cyp51A* gene mutations) from WT strains.[32] However, in the same study, a high percentage of isolates for which CLSI posaconazole MICs were 0.25 μg/ml harbored *cyp51A* gene mutations. In a murine invasive aspergillosis and PD target model, the posaconazole clinical MIC threshold was between 0.25 and 0.5 μg/ml or the same values as for the four WT isolates evaluated.[28] This apparent mismatch between phenotype and genotype may well be an example of where certain genetic mutations do not have an impact on the phenotype as described in Section "Phenotype versus genotype". Table 7a also summarizes additional susceptibility endpoints as reported in other studies for voriconazole and isavuconazole versus *A. fumigatus* SC and for *Aspergillus terreus* SC versus voriconazole.[24,29,41–43] In two in vitro invasive pulmonary aspergillosis and PK/PD models (to simulate clinical voriconazole data) as well in a murine model of disseminate infection, drug efficacy correlated with voriconazole MICs between 0.25 and 0.5 μg/ml for the infecting *A. fumigatus* strains.[29,41,43] Both animal and clinical data indicated susceptible endpoints ~1 μg/ml for isavuconazole.[29] In the murine model of *A. terreus* SC, drug efficacy correlated with MICs of ≤1.0 μg/ml,[42] which is the CLSI ECV for this species and antifungal combination (Tables 7 and 7a). Overall, all these studies generally show alignment with the proposed CLSI triazole ECVs for *Aspergillus* spp., despite the fact that those animal models also include the drugs pharmacology. Comparable data are not available for either amphotericin B or caspofungin and/or other moulds.
- **Mucorales and *Fusarium***. Table 8 depicts CLSI amphotericin B and posaconazole ECVs for *Lichtheimia corymbifera*, *Mucor circinelloides*, *Rhizopus arrhizus*, and *Rhizopus microsporus* and a CLSI ECV of itraconazole for *Rhizopus arrhizus*; insufficient data

Table 7
Available epidemiologic cutoff values (ECVs) in μg/ml of amphotericin B, caspofungin, itraconazole, isavuconazole, posaconazole, and voriconazole for clinically relevant *Aspergillus* species complexes and the CLSI and EUCAST broth microdilution methods.[a]

Species complex (SC)	Agent[b]	Incubation[c]	MIC/MEC range[d]	Mode[d]	CLSI ECV ≥97.5%[e]	EUCAST visual ECV
A. flavus SC	AMB	48 h	0.03–8	1	4	–
	CAS*	24 h	0.01–≥32	0.06	0.5	–
	ITR	48 h	0.03–16	0.5	1	1
	ISA	48 h	0.06–2	0.5	1	2
	POS	48 h	0.03–16	0.06	0.5	–
	VOR	48 h	0.06–16	0.5	2	2
A. fumigatus SC	AMB	48 h	0.03–16	0.5	2	–
	CAS*	24 h	0.016–32	0.25	0.5	–
	ITR	48 h	≤0.03–16	0.5	1	1
	ISA	48 h	0.06–8	0.5	1	2
	POS	48 h	≤0.03–4	0.06	0.25/0.25	–
	VOR	48 h	0.03–16	0.25	1	1
A. niger SC	AMB	48 h	0.03–2	0.5	2	–
	CAS*	24 h	0.01–2	0.06	0.25	–
	ITR	48 h	0.03–16	1	4	4
	ISA	48 h	0.06–>8	1	4	4
	POS	48 h	0.06–2	0.5	2	–
	VOR	48 h	0.03–16	0.5	2	2
A. terreus SC	AMB	48 h	0.12–32	2	4	–
	CAS*	24 h	0.01–2	0.06	0.12	–
	ITR	48 h	0.03–1	0.25	2	0.5
	ISA	48 h	0.06–2	0.25	1	2
	POS	48 h	0.03–2	0.25	1	–
	VOR	48 h	0.03–≥32	0.5	2	2
A. versicolor SC	AMB	48 h	0.03–8	1	2	–

[a] CLSI ECVs were defined using pooled MIC/MEC distributions for ≥100 isolates and from ≥3 laboratories by the CLSI document M38-A2 methodology.[3,8–10,13]
[b] AMB, amphotericin B; CAS, caspofungin; ITR, itraconazole; ISA, isavuconazole; POS, posaconazole; VOR, voriconazole.
[c] Incubation time for MIC and MEC (*__caspofungin MEC only__) determination as per M38-A2 document.[3]
[d] MIC range and mode (most frequent MIC/MEC) for pooled distributions used for the definition of each set of ECVs.[8–10,13]
[e] Calculated ECVs using the iterative statistical method and comprising >97.5% of the statistically modeled population as per CLSI criteria.[8–10,13] EUCAST ECVs were set by visual examination of the data (http://mic.eucast.org/Eucast2/).[23]

Table 7a
Other proposed triazole susceptibility endpoints by using different approaches for *A. fumigatus* SC and *A. terreus* SC.

Species complex (SC)	Agent	Model	Target	Endpoint	Ref.
A. fumigatus SC	Itraconazole Posaconazole Voriconazole	CLSI MIC distributions: WT and mutant strains CLSI MICs	Separation WT and Non-WT	ECVs: 1 μg/ml (itraconazole) 0.12 μg/ml (Posaconazole) 1 μg/ml (voriconazole)	32
A. fumigatus SC	Posaconazole	Murine IPA WT MICs: 0.25–0.5 μg/ml Mutant MICs: 2–8 μg/ml	Current static dose PD	Threshold MIC: 0.25–0.5 μg/ml	28
A. fumigatus SC	Voriconazole	In vitro IPA model In vitro PK/PD Murine disseminated infection	AUC/MIC = 55 Optimum treatment target Reduced fungal load Prolonged survival %	Max MIC: ≤0.5 μg/ml MIC: ≤0.25 μg/ml MIC: ≤0.25 μg/ml	24 43 41
A. fumigatus SC	Isavuconazole	Murine neutropenic IPA AUC/MIC WT MICs: ≤1 μg/ml Mutant MICs: 0.12–8 μg/ml	Outcome correlation PD target	14 day survival: ≤0.5 μg/ml (Better predictor value than mutations)	29
	Isavuconazole	Baseline isolates: clinical trial *A. fumigatus*: 28 A. flavus: 7 *A. terreus*: 6 MICs: 0.25 to ≥4 μg/ml	PK/PD data: Clinical efficacy	SBP: ≤1 μg/ml[a]	Not available
A. terreus SC	Voriconazole	Murine disseminated infection MICs: 0.12 to ≥4 μg/ml	Efficacy	Best survival: ≤1 μg/ml	42

WT, wild type (no *cyp51* gene mutations); non-WT, mutants or harboring *cyp51* gene mutations; IPA, invasive pulmonary aspergillosis; PD, pharmacodynamic parameters; PK, pharmacokinetic parameters; AUC, area under the curve; AUC/MIC, PD target for best outcome;
[a] SBP, susceptible BP (as presented but not approved by the CLSI, January 2015).

Table 8
Available epidemiologic cutoff values (ECVs) in μg/ml of amphotericin B, itraconazole, and posaconazole for four species of Mucorales and the CLSI broth microdilution method.[a]

Species	Agent[b]	MIC range[c]	Mode[c]	ECV ≥ 97.5%[d]
L. corymbifera	AMB	0.06–16	0.5	2
	ITR	0.06–4	0.25	–
	POS	0.06–8	0.5	2
M. circinelloides	AMB	0.03–4	0.25	2
	ITR	0.06–16	1	–
	POS	0.25–16	4	4
R. arrhizus	AMB	0.03–4	1	4
	ITR	0.03–32	0.5	2
	POS	0.06–16	0.5	2
R. microsporus	AMB	0.06–4	0.5	2
	ITR	0.06–16	0.5	–
	POS	0.25–32	1	2

[a] CLSI ECVs were defined using pooled MIC distributions for ≥100 isolates and from > 3 laboratories by the CLSI document M38-A2 methodology.[3,17] Incubation time for MIC determination as per M38-A2 document, 24 h.[3]
[b] AMB, amphotericin B; ITR, itraconazole; POS, posaconazole.
[c] MIC range and mode (most frequent MIC) for pooled distributions used for the definition of each set of ECVs.[17]
[d] Calculated ECVs using the iterative statistical method and comprising ≥97.5% of the statistically modeled population as per CLSI criteria.[17]

Table 9
Available epidemiologic cutoff values (ECVs) in μg/ml of amphotericin B, itraconazole, posaconazole, and voriconazole for two clinically relevant *Fusarium* species complexes and *F. verticillioides* and the CLSI broth microdilution method.[a]

Species complex (SC) or species	Agent[b]	MIC range[c]	Mode[c]	ECV ≥ 97.5%[d]
F. verticillioides	AMB	0.5–16	2	4
	ITR	1–≥16	16	–
	POS	≤0.25–≥16	0.5	2
	VOR	0.5–≥16	2	4
F. oxysporum SC	AMB	≤0.25–16	2	8
	ITR	1–≥16	16	32
	POS	0.5–16	2	8
	VOR	0.5–≥16	4	16
				8
F. solani SC	AMB	≤0.25–16	2	8
	ITR	0.5–≥16	16	32
	POS	1–≥16	8	32
	VOR	0.5–≥16	8	32

[a] CLSI ECVs were defined using pooled distributions for ≥100 isolates and from >3 laboratories by the CLSI document M38-A2 methodology.[3,19]
[b] AMB, amphotericin B; ITR, itraconazole; POS, posaconazole; VOR, voriconazole.
[c] MIC range and mode (most frequently MIC) for pooled distributions used for the definition of each set of ECVs.[19]
[d] Calculated ECVs using the iterative statistical method and comprising ≥97.5% of the statistically modeled population as per CLSI criteria.[19]

for the latter species precluded calculation of ECVs for the other two agents.[17] Table 9 lists recently reported CLSI ECVs of *Fusarium verticillioides*, *Fusarium oxysporum* SC, and *Fusarium solani* SC and amphotericin B, itraconazole and posaconazole; again insufficient data precluded the calculation of an ECV of itraconazole for *F. verticillioides*.[19] All isolates included in both studies were identified by currently accepted molecular methodologies to either the species or SC level. Again, no genetic information is available for these species/agents combinations. As previously stated in Section "ECVs: Their potential use in clinical practice", some of the ECVs, especially those for *Fusarium* spp., are quite high and suggest that they would be unresponsive to treatment with the relevant antifungal agent. It is important to emphasize again that, especially for the latter species, a categorization of an isolate as WT is not equivalent to being susceptible (treatable). CLSI ECVs have not been calculated for any other mould/agent combination, but useful MIC distributions and modes for less prevalent

Table 10
CLSI and other epidemiologic cutoff values (ECVs) in μg/ml of amphotericin B, anidulafungin, caspofungin, micafungin, fluconazole, itraconazole, posaconazole, and voriconazole for clinically relevant *Candida* species/complexes and the CLSI, EUCAST and SYO broth microdilution methods.[a]

Species or species complex (SC)	Agent[b]	MIC range[c]	Mode[c] CLSI/SYO	CLSI-Based ECV ≥97.5%[d]	SYO ECV ≥97.5%[d]	EUCAST Visual ECV	SBP[e]
C. albicans	AMB	≤0.03–4	0.5	**2**	–	1	–
	ANI	0.008–2	0.03/0.03	**0.12**	0.12	0.03	≤0.12
	CAS	0.008–≥8	–/0.06	–	0.25	–	≤0.25
	MIC	0.008–4	0.01/0.008	**0.06**	0.03	0.01	≤0.25
	FLU	0.06–≥128	0.12	0.5	–	1	≤2
	ITR	–	–	–	–	0.06	–
	POS	0.008–≥8	0.01	0.03(W)/0.0.06(UW)	–	–	–
	VOR	0.008–≥8	0.01	0.03	–	0.12	≤0.12
C. dubliniensis	AMB	–	–	–	–	–	–
	ANI	0.01–4	0.03/0.12	**0.12**	0.25	–	–
	CAS	0.01–≥8	–/0.06	NA	0.25	–	–
	MIC	0.008–≥8	0.06/0.06	**0.12**	0.12	–	–
	FLU	0.06–64	0.25	0.5	–	–	–
	ITR	–	–	–	–	0.06	–
	POS	0.008–0.5	0.03	0.25	–	–	–
	VOR	0.008–1	0.01	0.03	–	–	–
C. glabrata SC[f]	AMB	0.03–4	1	**2**	–	1	–
	ANI	0.008–4	0.03/0.06	**0.12**	0.12	0.06	≤0.12
	CAS	0.008–≥8	–/0.12	–	0.25	–	≤0.12
	MIC	0.008–4	0.01/0.01	**0.03**	0.03	0.03	≤0.06
	FLU	0.12–≥128	4	8	–	32	–
	ITR	≤0.01–>8	0.5	**4**	–	2	–
	POS	0.008–≥8	0.25	1(W)2(UW)	–	–	–
	VOR	0.008–≥8	0.06	0.5(W)0.25(UW)	–	1	–
C. guilliermondii	AMB	–	–	–	–	–	–
	ANI	0.03–4	1/1	**8**	4	–	≤2
	CAS	0.06–≥8	–/0.5	–	2	–	≤2
	MIC	0.01–8	0.5/0.5	**2**	2	–	≤2
	FLU	0.12–64	2	**8**	–	16	–
	ITR	–	–	–	–	2	–
	POS	0.008–2	0.12	1(W)0.5(UW)	–	–	–
	VOR	0.008–≥8	0.03	0.**12**	–	0.25	–
C. lusitaniae	AMB	–	–	–	–	–	–
	ANI	0.008–1	0.25/-0.12	**1**	0.25	–	–
	CAS	0.008–1	–/0.25	–	2	–	–
	MIC	0.008–≥16	0.25/0.06	**0.5**	0.12	–	–
	FLU	0.12–64	0.5	1	–	16	–
	ITR	0.008–1	0.12	1	–	2	–
	POS	0.008–1	0.01	0.06	–	–	–
	VOR	0.008–0.25	0.01	0.03(W)0.06(UW)	–	0.25	–
C. krusei	AMB	0.03–4	1	**2**	–	1	–
	ANI	0.008–2	0.06	**0.25**	0.25	0.06	≤0.25
	CAS	0.03–≥8	–/0.25	–	1	–	≤0.25
	MIC	0.01–1	0.06	**0.25**	0.25	0.25	≤0.25
	FLU	0.25–≥128	16	32	–	128	–
	ITR	≤0.01–2	0.25	**1**	–	1	–
	POS	0.01–4	0.25	1(W)0.5(UW)	–	–	–
	VOR	0.008–2	0.12	0.5	–	1	≤0.5
C. parapsilosis SC	AMB	0.03–4	1	**2**	–	1	–
	ANI	0.008–8	2	**8**	4	4	≤2
	CAS	0.008–≥8	–/0.5	–	2	–	≤2
	MIC	0.01–4	1	**4**	4	2	≤2
	FLU	0.06–≥128	0.5	1	–	2	≤2
	ITR	–	–	–	–	0.12	–
	POS	0.008–2	0.03	0.06(W)0.25(UW)	–	–	–
	VOR	0.008–2	0.01	0.03	–	0.12	≤0.12
C. tropicalis	AMB	0.03–4	1	**2**	–	1	–
	ANI	0.008–4	0.03	**0.12**	0.5	0.06	≤0.25
	CAS	0.008–≥8	0.06	–	0.25	–	≤0.25
	MIC	0.008–8	0.01	**0.06**	0.06	0.06	≤0.25
	FLU	0.06–≥128	0.25	1	–	2	≤2
	ITR	≤0.01–8	0.12	**0.5**	–	0.12	–
	POS	0.008–≥8	0.03	0.12	–	–	–
	VOR	0.008–≥8	0.01	0.06	–	0.12	≤0.12

[a] CLSI and SYO ECVs were defined using pooled distributions for ≥100 isolates and from ≥3 laboratories by the CLSI document M27-A3 and/or SYO methodologies.[4,15,18,38,39]

[b] AMB, amphotericin B; ANI, anidulafungin; CAS, caspofungin, MIC, micafungin; FLU, fluconazole; ITR, itraconazole; POS, posaconazole; VOR, voriconazole.

[c] MIC range and mode (most frequently MIC) for pooled distributions used for the definition of each set of ECVs.[15,18,38,39]

[d] Calculated ECVs using the iterative statistical method and comprising ≥97.5% of the statistically modeled population as per CLSI criteria.

W (weighted) and UW (unweighted) ECV: At least one of the distributions comprised ≥50% of the pooled values which led to the need for the weighting analysis (See Table 3). See criterion xi regarding the unresolved issue when the values are different following normalization of the data (W analysis).

[e] SBP, available susceptible BPs.

[f] SBPs are not available for the combination of *C. glabrata* SC and fluconazole, only susceptible dose dependent (≤ 32 μg/ml) and resistant BPs (≥ 64 μg/ml) are listed in the CLSI document.[6]

ECVs are not available for flucytosine and various species and itraconazole due to truncated distributions and/or insufficient data as per CLSI criteria.

EUCAST ECVs were set by visual examination of the data (http://mic.eucast.org/Eucast2/).

Mucorales and *Fusarium* spp. can be found in references 17 and 19.

- ***Candida***. Given that *Candida* cause the majority of severe fungal infections, more information has accumulated regarding MIC distributions, molecular mechanisms of resistance, and correlation of in vitro versus in vivo results from formal clinical trials. Because of that, BPs are available for more prevalent *Candida* spp. and two triazoles (fluconazole and voriconazole) and echinocandins.[6,35–37] ECVs also have been defined for less prevalent species, including ECVs of amphotericin B and itraconazole for various prevalent *Candida* spp.[15,38,39] Table 10 depicts CLSI approved, ECV (**bolded**) and the parallel available SBP (right column) for eight *Candida* spp. Due to interlaboratory variation (high degree of modal differences among 14 laboratories) of CLSI caspofungin MICs for *Candida* spp., the definition of CLSI-ECVs for *Candida* spp. and caspofungin was precluded (Table 2).[14] **When BPs have been established for the species/agent combination evaluated, the value that should be used is the BP.** Visual EUCAST ECVs and SYO ECVs are also incorporated in Table 10.
- ***Cryptococcus***. CLSI ECVs of seven agents used or potentially useful in the treatment of cryptococcal infections also have been calculated for *Cryptococcus neoformans* (including molecular type VNI) and *Cryptococcus gattii* (including molecular types VGI and VGII; Table 11).[11,12,16] Although data were available in those studies for other molecular types (VNII, VNIII and VNIV and VGIIb, VGIIc, VGIII and VGIV), insufficient data (<100 isolates and/or originating in only two laboratories) precluded ECV calculation; however, their MIC distributions and modes can be found in references 11, 12, and 16. It is noteworthy that VGII and VGIII genotypes can include at least three and two subtypes, respectively (VGIIa, VGIIb, and VGIIc and VGIIIa and VGIIIb). Given that BPs are not available for this important fungal group, these CLSI ECVs can be useful since the incidence of infections caused by these two species complexes continues to increase.
- **Method-dependent SYO-based ECVs**. Method-dependent ECVs based on Sensititre® YeastOne® (SYO) susceptibility testing data for several species of *Candida* spp. and the echinocandins have been published and are listed in Table 10.[18] Since no major inter-laboratory variation in caspofungin MIC distributions was evident with SYO panels, it has been possible to estimate method-dependent ECVs for this agent.[18] When echinocandin SYO and CLSI ECVs are available for the same species, modes and MIC ranges by both methods are listed; similar SYO MIC information for less prevalent *Candida* spp. are found in reference 18. In general, CLSI and SYO ECVs of anidulafungin and micafungin ECVs are the same or only one dilution different with several exceptions, some ECVs for *Candida lusitaniae*, *Candida tropicalis* and *Candida parapsilosis*. These differences emphasize the importance of using the interpretive endpoint for which it was established.

ECVs established for the EUCAST reference methods

EUCAST's reference methods for antifungal susceptibility testing differ in a small number of ways from those by CLSI methods (http://www.eucast.org/ast_of_fungi/methodsinantifungalsusceptibilitytesting/susceptibility/testing of yeasts/), sufficiently in some cases to yield different MICs/MECs.[20] ECVs by both methods tend to be similar, mostly two-fold dilutions of each other, with the differences attributable to differences in testing methods and/or ECV estimation (Tables 7 and 10). EUCAST ECVs for a number of organism/antifungal combinations using visual inspection are published on the EUCAST wild-type distribution website (http://mic.eucast.org/Eucast2/).

Table 11
Available epidemiologic cutoff values (ECVs)[a] in μg/ml of amphotericin B, flucytosine, fluconazole, isavuconazole, itraconazole, posaconazole, and voriconazole for *Cryptococcus neoformans-Cryptococcus gattii* species complex and the CLSI broth dilution microdilution method.

Species or molecular type	Agent[b]	MIC range[c]	Mode	ECV ≥ 97.5%[d]
C. neoformans	AMB	≤0.03–4	0.25	1
Non-typed	FCT	0.12–≥64	4	16
	FLU	≤0.12–≥64	4	16
	ISA	0.008–0.5	0.03	0.12
	ITR	0.01–≥4	0.25	0.5
	POS	0.01–4	0.12	0.5
	VOR	0.008–≥4	0.06	0.25
VNI	AMB	≤0.03–2	0.25	0.5
	FCT	0.12–64	4	8
	FLU	≤0.12–64	4	8
	ISA	0.008–0.5	0.03	0.12
	ITR	0.008–0.5	0.12	0.25
	POS	0.008–0.5	0.06	0.12 (W)/0.25(UW)
	VOR	0.008–4	0.06	0.25
C. gattii	AMB	0.06–1	0.5	1
Non-typed	FCT	0.25–8	1	–
	FLU	0.5–≥64	4	8
	ISA	0.008–0.5	0.03	0.25
	ITR	0.016–0.5	0.12	–
	POS	0.008–1	0.12	–
	VOR	0.008–0.5	0.06	–
VGI	AMB	0.03–1	0.25	0.5
	FCT	0.12–64	2	4
	FLU	0.25–16	4	16 (W/UW)
	ISA	–	–	–
	ITR	0.008–1	0.25	0.5 (W/UW)
	POS	0.016–1	0.12	–
	VOR	0.008–0.25	0.12	0.5 (W/UW)
VGII	AMB	0.06–1	0.25	1
	FCT	0.25–≥64	2	32
	FLU	1–≥64	8	32
	ISA	–	–	–
	ITR	0.03–1	0.12	1
	POS	–	–	–
	VOR	0.01–0.5	0.12	0.5

[a] ECVs were defined using pooled distributions for ≥100 isolates and from ≥3 laboratories by the CLSI document M27-A3 methodology.[4,11,12,16]

[b] AMB, amphotericin B; FCT, flucytosine; FLU, fluconazole; ISA, isavuconazole; ITR, itraconazole; POS, posaconazole; VOR, voriconazole.

[c] MIC range and mode (most frequent MIC) for pooled distributions used for the definition of each set of ECVs.[11,12,16]

[d] Calculated ECVs using the iterative statistical method and comprising ≥97.5% of the statistically modeled population as per CLSI criteria.[1,11,12]
W (weighted) and UW (unweighted) analysis, respectively: At least one of the distributions comprised ≥50% of the pooled values which led to the need for the weighting analysis (See Table 3).

Conclusions

For the first same time in the field of antifungal chemotherapy, interpretive endpoints (BPs and ECVs) are available for the more prevalent yeast and mould species that cause serious infections, especially in the immuno-compromised host. Although the new interpretive categories (ECVs) do not predict clinical response as BPs do, they would identify non-WT or less susceptible isolates. These non-WT isolates are less likely to respond to therapy. Since excessive variability is still present in reference method susceptibility testing of caspofungin for *Candida* spp., SYO ECVs are now available for this widely used commercial assay. As CLSI supplements may be revised yearly, it is expected that more of these values would be listed in future versions of these supplements as well as some of the listed ECVs in the present could be modified as new data or taxonomic issues are resolved. It is hoped that in the near future, EUCAST will provide ECVs that have been calculated in the similar statistically manner as those for the CLSI methods and

that ECVs for other species and agent combinations as well as for the Etest also will be available.

Conflict of interest

The authors declare no conflict of interest.

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Annex 1.

List of the principal investigators who provided each single distribution for the estimation of each ECV.[a]

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