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Wild-Type MIC Distributions and Epidemiological Cutoff Values for Amphotericin B and *Aspergillus* spp. for the CLSI Broth Microdilution Method (M38-A2 Document)▽

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Although clinical breakpoints have not been established for mold testing, epidemiological cutoff values (ECVs) are available for *Aspergillus* spp. versus the triazoles and caspofungin. Wild-type (WT) MIC distributions (organisms in a species-drug combination with no acquired resistance mechanisms) were defined in order to establish ECVs for six *Aspergillus* spp. and amphotericin B. Two sets (CLSI/EUCAST broth microdilution) of available MICs were evaluated: those for *A. fumigatus* (3,988/833), *A. flavus* (793/194), *A. nidulans* (184/69), *A. niger* (673/140), *A. terreus* (545/266), and *A. versicolor* (135/22). Three sets of data were analyzed: (i) CLSI data gathered in eight independent laboratories in Canada, Europe, and the United States; (ii) EUCAST data from a single laboratory; and (iii) the combined CLSI and EUCAST data. ECVs, expressed in μg/ml, that captured 95%, 97.5%, and 99% of the modeled wild-type population (CLSI and combined data) were as follows: for *A. fumigatus*, 2, 2, and 4; for *A. flavus*, 2, 4, and 4; for *A. nidulans*, 4, 4, and 4; for *A. niger*, 2, 2, and 2; for *A. terreus*, 4, 4, and 8; and for *A. versicolor*, 2, 2, and 2. Similar to the case for the triazoles and caspofungin, amphotericin B ECVs may aid in the detection of strains with acquired mechanisms of resistance to this agent.

In addition to *Aspergillus fumigatus*, other *Aspergillus* species, such as *A. flavus*, *A. niger*, and *A. terreus*, have increased as the cause of severe opportunistic infections, especially in the immunocompromised host (4, 11, 24). The mortalities for untreated and treated patients with invasive aspergillosis are practically 100 and 50%, respectively (5, 20, 27). Since the introduction in 1956 of amphotericin B, the polyenes (conventional amphotericin B, liposomal, and lipid complex) have been used for the systemic treatment of yeast and mold infections (empirical, primary, or salvage therapy against aspergillosis and other invasive infections), especially in neutropenic febrile patients (11, 34).

Most *Aspergillus* isolates are considered susceptible to amphotericin B, but *A. terreus* seems to be intrinsically resistant to this agent (13, 17, 29, 35), and high MICs (>1 μg/ml) also have been reported among isolates of *A. flavus* (13, 26), *A. lentulus* (1, 4), *A. nidulans* (13), *A. ustus* (21), and even *A. fumigatus* (13, 19). Similar to the case for caspofungin, the mechanisms of polyene activity and resistance in *Aspergillus* spp. have not been clearly determined. The polyenes act by forming pores in the fungal membrane that lead to membrane leakage and possible fungal cell death (33); fungal oxidative cellular damage also has been reported in *Candida albicans* (28).

The availability of reference methodologies (8, 9) has enabled the development of Clinical and Laboratory Standards Institute (CLSI) epidemiological cutoff values (ECVs) for various *Aspergillus* spp. versus the triazoles (14, 22) and caspofungin (15); ECVs are also available for *A. fumigatus* and the triazoles (25) by use of the Subcommittee on Antifungal Susceptibility Testing of the European Committee for Antimicrobial Susceptibility Testing (AFST-EUCAST) methodology (30). Abundant amphotericin B data are available for *Aspergillus* spp. (1–4, 13, 17, 19, 29, 35), but neither MIC distributions nor ECVs are available for any polyene and mold combination. The frequent use of amphotericin B and its lipid formulations continues to increase selection pressure, and monitoring of emerging polyene resistance in *Aspergillus* spp. is therefore important.

The purpose of the study was to define wild-type (WT) susceptibility endpoint distributions (see "Definitions" below) of each *Aspergillus* spp. and amphotericin B by using aggregated CLSI MIC data gathered in eight laboratories in Canada, Europe, and the United States (135 to 3,988 MICs according to the species) and EUCAST data from a single laboratory (22 to 833 MICs according to species); we used these data to propose amphotericin B ECVs (see "Definitions" below) for each species (*A. fumigatus*, *A. flavus*, *A. nidulans*, *A.*

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TABLE 1. MICs of amphotericin B for QC and reference strains as determined in eight laboratories at 48 h

QC or laboratory control isolate	QC MIC range (mode),[a] μg/ml	% CLSI MICs within range	MIC range (mode),[a] μg/ml, determined by laboratory:							
			1	2	3	4	5	6	7	8
Paecilomyces variotii ATCC MYA-3630	1–4 (2)	100	ND	1–2 (2)	1–2 (2)	ND	1–4 (2)	ND	ND	1–2 (2)
Aspergillus flavus ATCC 204304	0.5–4 (NA)	100	0.5–1 (0.5)	ND	ND	0.25–1 (1)[b]	ND	ND	0.5–2 (2)	ND
Aspergillus fumigatus NCPF 7097	NA	NA	ND	ND	ND	ND	ND	0.5–2 (1)	ND	ND
Aspergillus fumigatus NCPF 7100	NA	NA	ND	ND	ND	ND	ND	1–2 (1)	ND	ND
Candida parapsilosis ATCC 22019	0.5–4 (2)	91.7	0.5 (0.5)	1–2 (2)	0.5–4 (1)	0.5–2 (1)	ND	0.5–4 (1)	0.5–1 (1)	ND
Candida krusei ATCC 6258	1–4 (2)	100	1 (1)	1–4 (2)	0.5–2 (1)	1–2 (2)	1–4 (2)	1–2 (1)	1–2 (2)	1–2 (2)

[a] MICs were determined as recommended by the CLSI M38-A2 document (8). ND, Not determined. NA, QC MIC limits are not available, but the *A. flavus* strain is listed in the CLSI M38-A2 document as a reference isolate.
[b] MICs were outside the range for reference isolate *A. flavus* ATCC 204304 (2%).

niger, *A. terreus*, and *A. versicolor*). In the absence of clinical breakpoints, ECVs could help to characterize the susceptibility of *Aspergillus* sp. isolates to amphotericin B and its lipid formulations and to monitor the emergence of strains with mutations which could lead to reduced polyene susceptibility.

MATERIALS AND METHODS

Isolates. Each isolate was recovered from a unique clinical specimen at eight medical centers: the University of Texas Health Science Center, San Antonio, TX; the HPA Mycology Reference Laboratory, Kingsdown, Bristol, United Kingdom; Hospital General Universitario Gregorio Marañón, Madrid, Spain; Servicio de Micología, Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Spain; the University of Iowa, Iowa City, IA; the University Hospitals of Cleveland, Cleveland, OH; the University of Alberta Hospital, Edmonton, Canada; and VCU Medical Center, Richmond, VA. Isolates were identified and stored at each medical center using standardized methodologies; isolates were not genetically characterized for mutations. The total numbers of aggregated available CLSI MIC data from the eight laboratories per species were as follows: 3,988 isolates of *A. fumigatus*, 793 isolates of *A. flavus*, 184 isolates of *A. nidulans*, 673 isolates of *A. niger*, 545 isolates of *A. terreus*, and 135 isolates of *A. versicolor*. The total available EUCAST MIC data from a single laboratory (Instituto de Salud Carlos III, Majadahonda, Spain) per species were as follows: 833 isolates of *A. fumigatus*, 194 isolates of *A. flavus*, 69 isolates of *A. nidulans*, 140 isolates of *A. niger*, 266 isolates of *A. terreus*, and 22 isolates of *A. versicolor*.

Three quality control (QC) isolates, *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, and *Paecilomyces variotii* ATCC MYA-3630, and three reference molds, *A. flavus* ATCC 204304 and *A. fumigatus* NCPF 7100 and NCPF 7097, were used by the participant laboratories (8). Table 1 shows the CLSI MIC data for these isolates; EUCAST data from the single laboratory were similar for both QC yeasts and the *A. flavus* strain (mostly 1 dilution lower).

Antifungal susceptibility testing. In order to include MIC results in the set of CLSI aggregated data from the eight laboratories (Table 2), amphotericin B MICs were obtained at each center by following the CLSI M38-A2 broth microdilution method (8). The testing parameters for determining EUCAST MIC data were similar to those for the CLSI method except that the EUCAST RPMI 1640 broth contained 2% versus 0.2% dextrose, the inoculum concentration was $\sim 10^5$ CFU/ml versus $\sim 10^4$ CFU/ml, and the final dimethyl sulfoxide (DMSO) concentration was 0.5% versus 1% (30). At least two of the three QC strains and/or one of the reference strains were utilized during the years of testing in each center.

Definitions. The WT population is the subpopulation of isolates/MICs in a species-drug combination with no detectable acquired resistance mechanisms (10, 31). The highest WT susceptibility endpoint has been defined as either the WT cutoff value (CO_{WT}) or the ECV, which is the critical drug concentration that may identify those strains with decreased susceptibility or serve as an early warning of emerging subtle changes in the patterns of susceptibility of organisms to the agent being evaluated (10, 31). The latter term has been used previously in similar reports on fungi (14, 15, 22, 23, 25).

Data analysis. Three MIC distributions for each species were obtained: (i) CLSI aggregated data from the eight laboratories, (ii) single-laboratory EUCAST MIC data, and (iii) the combined CLSI and EUCAST data. The MIC distribution of each species from each laboratory was reviewed for outlier results, and modal MICs were determined for species from each laboratory; ECVs were determined for each distribution and species by a previously reported statistical technique (31). Briefly, the modeled population is based on fitting a normal distribution to increasing subsets of the data starting at that population that includes isolates with MICs 1 dilution higher than the mode (or than the lower mode if there is more than one mode) and working out the mean and standard deviation (SD) of the cumulative normal distribution that best fits those data;

TABLE 2. Wild-type (WT) MIC distributions of amphotericin B for six *Aspergillus* spp. from eight laboratories, obtained using CLSI M38-A2 and EUCAST microdilution methods

Species	Total	No. of isolates tested by CLSI/EUCAST method[a] With MIC (μg/ml) of:										
		≤0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	16	32
A. fumigatus	3,988/833	13/0	56/5	323/73	600/447[b]	1,389[b]/245	1,382/53	214/7	9/1	2/1	0/1	0/0
A. flavus	793/194	7/0	3/0	8/1	63/11	141/72	363[b]/76[b]	187/27	19/6	2/1	0/0	0/0
A. nidulans	184/69	0/0	1/0	2/1	9/5	23/22	68[b]/21[b]	70/12	9/4	2/1	0/1	0/2
A. niger	673/140	2/0	43/5	38/55	122/71[b]	252[b]/9	194/0	12/0	0/0	0/0	0/0	0/0
A. terreus	545/266	0/0	0/0	3/0	10/35	21/37	109/104[b]	297[b]/86	84/22	20/9	1/4	0/1
A. versicolor	135/22	1/0	1/0	4/1	3/2	13/2	76[b]/9[b]	26/3	10/1	1/0	0/0	0/0

[a] Number of isolates tested by the CLSI and EUCAST broth microdilution methods (8, 30), including CLSI aggregated data from eight laboratories and EUCAST data from a single laboratory.
[b] MIC most frequently obtained by each broth microdilution method.

TABLE 3. ECVs for amphotericin B and six *Aspergillus* spp., obtained using CLSI M38-A2 and/EUCAST broth microdilution methods at 48 h

Species	No. of isolates tested by CLSI/EUCAST method[a]	MIC (μg/ml):		Calculated statistical ECV (μg/ml)[d]		
		Range[b]	Mode[c]	≥95%	≥97.5%	≥99%
A. fumigatus	3,988/833	0.032–16	0.5/0.25/0.5	2/0.5/2[e]	2/1/2	4/1/4
A. flavus	793/194	0.032–8	1/1/1	2/2/2	4/2/4	4/2/4
A. nidulans	184/69	0.06–32	1/1/1	4/2/4	4/4/4	4/4/8
A. niger	673/140	0.03–2	0.5/0.25/0.5	2/0.5/2[e]	2/0.5/2	4/0.5/4
A. terreus	545/266	0.12–32	2/1/2	4/4/4	4/4/4	8/4/8
A. versicolor	135/22	0.032–8	1/1/1	2/2/2	2/4/2	2/4/4

[a] CLSI aggregated data from eight laboratories and EUCAST data from a single laboratory.
[b] Combined CLSI and EUCAST data.
[c] MIC most frequently obtained using CLSI/EUCAST/combined CLSI and EUCAST data.
[d] Calculated ECVs comprising ≥95, ≥97.5, or ≥99% of the statistically modeled population, obtained using CLSI/EUCAST/combined CLSI and EUCAST data.
[e] The ECV defined using the "eyeball" method, which included at least 95% of the observed (rather than the modeled) overall distribution, was 1 μg/ml.

those numbers are used to calculate the MIC values that capture at least 95%, 97.5%, and 99% of the modeled WT population. The MIC values that captured greater than or equal to 95% of the observed population ("eyeball" method) were also calculated. The latter method has been used previously to define ECVs for azoles and echinocandins (14, 15, 22, 23). Only 95% and 99% cutoffs were calculated in a previous study (15), while in this study the 97.5% value was calculated, as increasing experience has shown that this agrees most often with values set using the "eyeball" method (14, 15).

RESULTS AND DISCUSSION

The value of susceptibility testing is optimal when the test result can define the likely response to treatment of infections caused by the organism being tested against a specific agent. Clinical breakpoints are supposed to predict the outcome of therapy and are based on clinical trial data, global susceptibility surveillance, resistance mechanisms, and pharmacokinetic/pharmacodynamic parameters from model systems (10, 32). Although a correlation between high azole MICs and poor treatment responses has been reported, no clear correlation has been found for *Aspergillus* spp. and high amphotericin B MICs (>1 μg/ml), except for *A. terreus* (29, 35). In addition, the low number of mold infections and the scarcity of isolates with high antifungal susceptibility endpoints that might predict failure have precluded the establishment of clinical breakpoints for mold testing. However, CLSI ECVs for several *Aspergillus* spp. versus the triazoles and caspofungin (14, 15, 22) and EUCAST ECVs for *A. fumigatus* and the triazoles (25) are available. In the present study, amphotericin B ECVs for six *Aspergillus* spp. were defined. These ECVs should aid in the evaluation of clinical isolates by identifying those strains that may have acquired resistance mechanisms to amphotericin B and should serve as an early warning of emerging subtle changes in the susceptibility patterns of these fungal pathogens.

Variability is expected when MICs from different laboratories are compared, despite standardization efforts; interlaboratory variation has been observed even under controlled conditions with single strains in QC or in other collaborative studies (8, 9, 12). In our study, each laboratory provided CLSI MIC data for one or both QC isolates, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, when clinical isolates were tested. Additional MIC data were provided by some laboratories for either the QC *P. variotii* MYA-3630 isolate, the reference isolate *A. flavus* ATCC 204304, or their respective internal mold controls (Table 1). Most MIC ranges were within the CLSI established limits (98 to 100%) for the three QC and reference *A. flavus* ATCC 204304 isolates (8), but the modes were variable (mostly ± one 2-fold dilution) for the QC yeast and reference *A. flavus* ATCC 204304 isolates. The intralaboratory reproducibility also was excellent (31 replicate tests) in the laboratory that used the two strains of *A. fumigatus* (NCPF 7100 and 7097). The modal MIC variability among the participant laboratories for control isolates reflected the modal MIC differences for the clinical *Aspergillus* isolates. This phenomenon has been observed in previous ECV studies for triazoles and caspofungin (14, 15) and may reflect individual interpretations of susceptibility endpoints. The MIC data for the two QC yeast isolates obtained by the EUCAST methodology were also within the established ranges.

Table 2 shows both sets of amphotericin B MIC distributions for the six *Aspergillus* spp. (CLSI aggregated MIC data/single-laboratory EUCAST data). Table 3 depicts the three modal MICs (aggregated CLSI/EUCAST and combined CLSI and EUCAST data) for each of the six *Aspergillus* spp. The lowest modal MICs (0.25 to 0.5 μg/ml for EUCAST and CLSI data, respectively) were for *A. fumigatus* and *A. niger*. Either the other modal MICs (1 or 2 μg/ml) were the same or the EUCAST modal MIC was lower. Among individual contributing laboratories, modal MIC values were similar (equal to or within one 2-fold dilution) for all the species tested. Each CLSI amphotericin B modal MIC ± one 2-fold dilution comprised similar percentages of the populations: 84.4% of *A. niger* isolates, 84.5% of *A. fumigatus* isolates, 85.2% of *A. versicolor* isolates, 87.1% of *A. flavus* isolates, 87.5% of *A. nidulans* isolates, and 90% of *A. terreus* isolates. The percentages were similar when the two sets of data were combined and were higher with the EUCAST data for *A. fumigatus* (91.8%), *A. flavus* (90.2%), and *A. niger* (96.4%). Comparable data have been obtained in previous triazole and caspofungin ECV studies for *Aspergillus* spp. (14, 15). Therefore, based on these data and the wide geographical range over which MICs were collected for our study, we are confident of the validity of our MIC data.

Table 3 also depicts the proposed amphotericin B ECVs (using ≥95%, 97.5%, and ≥99% of the modeled MIC population). CLSI amphotericin B ECVs encompassing ≥95% of the modeled MIC population were either 2 μg/ml (*A. fumiga-*

TABLE 4. Percentages of isolates of six *Aspergillus* spp. above the amphotericin B WT distribution from eight laboratories as determined by CLSI M38-A2 and EUCAST broth microdilution methods at 48 h

Species	ECV, μg/ml (% of isolates above each WT distribution)[a]		
	≥95%	≥97.5%	>99%
A. fumigatus	2 (0.3)/0.5 (7.6)/2 (0.3)	2 (0.3)/1 (1.2)/2 (0.3)	4 (0.1)/1 (1.2)/4 (0.1)
A. flavus	2 (2.6)/2 (3.6)/2 (2.8)	4 (0.3)/2 (3.6)/4 (0.3)	4 (0.3)/2 (3.6)/4 (0.3)
A. nidulans	4 (1.1)/2 (11.6)/4 (2.4)	4 (1.1)/4 (5.8)/4 (2.4)	4 (1.1)/4 (5.8)/8 (1.2)
A. niger	2 (0)/0.5 (0)/2 (0)	2 (0)/0.5 (0)/2 (0)	4 (0)/0.5 (0)/4 (0)
A. terreus	4 (3.9)/4 (5.3)/4 (4.3)	4 (3.9)/4 (5.3)/4 (4.3)	8 (0.2)/4 (5.3)/4 (0.7)
A. versicolor	2 (8.1)/2 (4.5)/2 (4.5)	2 (8.1)/4 (0)/2 (7.6)	2 (8.1)/4 (0)/4 (0.6)

[a] Calculated ECVs comprising ≥95, ≥97.5, or ≥99% of the statistically modeled population, determined using CLSI/EUCAST/combined CLSI and EUCAST data.

tus, *A. flavus*, *A. niger*, and *A. versicolor*) or 4 μg/ml (*A. nidulans* and *A. terreus*). As for the modal MICs, ECVs obtained by the EUCAST method were lower for three of the six species (*A. fumigatus* [0.5 μg/ml], *A. niger* [0.5 μg/ml], and *A. nidulans* [2 μg/ml]), but ECVs were the same as the CLSI cutoffs when the two sets of data were combined (Table 3). It is noteworthy that when the ECV is defined using the "eyeball" technique (at least 95% of observed overall distribution), the CLSI ECV for *A. niger* is 1 μg/ml (encompassing 98.2% of the isolates) and then only 1 instead of 2 dilutions higher than the EUCAST cutoff. The discrepancy between the two ECV definition methodologies was observed only for this species. The statistical method models the MIC distribution and, after doing that, predicts that a small proportion (<2.5%) of the WT isolates will actually be above the ECV. Because of that, the statistically calculated ECV for *A. niger* is 2 μg/ml despite the fact that there were no observed values above the calculated cutoff. From the clinical point of view, some non-WT isolates could be "erroneously" categorized as WT using the statistical cutoff or, conversely, as non-WT using the "eyeball" value. The difference between ECVs defined using the single-laboratory EUCAST data and ECVs defined using the CLSI aggregated data was due mostly to data size and corresponding SDs. A larger SD can result in a higher estimated cutoff even if the means are the same, because the modeled population is "more spread out." On the other hand, the combined EUCAST and CLSI data had a very large number of isolates/MICs, which led to ECVs similar to those defined using the CLSI data (Table 3). Earlier comparison of the EUCAST and CLSI methods (with triazoles) or of their testing conditions for amphotericin B provided comparable results (7, 16). ECVs were mostly the same or 1 dilution higher when these values were encompassing either ≥97.5% or ≥99% of each modeled population.

The frequency of amphotericin B MICs above the ECV varied according to the species and the set of data analyzed (Table 4). There were no MICs above the ECV (non-WT) for *A. niger* (three data sets) and only 0.1 to 0.3% for *A. fumigatus* (CLSI data). The highest percentages of non-WT CLSI MICs were observed for *A. versicolor* (8.1%) and *A. terreus* (3.9%), followed by *A. flavus* (2.6%) and *A. nidulans* (1.1%) (ECVs encompassing ≥95% of the calculated populations). As expected, percentages of non-WT isolates with the EUCAST data were higher for most species (ECVs encompassing ≥95% of the calculated populations), especially for *A. fumigatus* (7.6 versus 0.3%) and *A. nidulans* (11.6 versus 1.1%), and lower for *A. versicolor* (4.5 versus 8.1%), but the number of EUCAST MICs/isolates for the last species was low (Tables 2 and 3). Similarly low amphotericin B MICs have been recently reported for *Aspergillus* section *Nigri*, including *A. niger* (MICs mostly <0.5 μg/ml) (2, 3), while the percentages of MICs that were >1 μg/ml for *A. terreus* are consistently high by both methods in this study (45.9% [EUCAST] and 73.8% [CLSI]) and other studies (≥68%) (3, 17). These latter results are in agreement with the poor response to the different formulations of amphotericin B in patient care and in experimental aspergillosis caused by *A. terreus* (17, 29, 35). Information on response rates is not available for *A. terreus* isolates with MICs of <1 μg/ml or for *A. fumigatus* isolates with MICs of >1 μg/ml. Recently, an increase in amphotericin B MICs during therapy was associated with no decrease in mortality at 12 weeks (versus a decrease at 6 weeks) in transplant patients with proven invasive aspergillosis (3). As in our study, high incidences of triazole (mostly itraconazole) and caspofungin non-WT isolates (≥5%) have been observed for *A. nidulans*, *A. versicolor*, and *A. flavus* (3, 14, 15). Collectively, these data highlight the fact that ECVs are species specific and cannot be merged if they are to act as surrogates for the early detection of acquired resistance.

Similar to the case for caspofungin and *Aspergillus* spp., the relationship between gene mutations and high amphotericin B MICs for *Aspergillus* spp. has not been determined. The little information available regarding the mechanisms of amphotericin B resistance is mostly for *Candida albicans*, *A. terreus*, and *A. flavus*. Alteration of cell wall composition has been found to be responsible for *A. flavus* resistance to this agent (26). In one *A. terreus* isolate (amphotericin B MIC, 16 μg/ml), the ergosterol content was lower than that in *A. fumigatus* isolates (amphotericin B MICs, ≤0.5 μg/ml) (35). More recently, ergosterol was found not to play a crucial role in *A. terreus* resistance to amphotericin B (mean ergosterol contents for *A. fumigatus* and *A. terreus* were 4.4 ± 1.4 and 5.1 ± 2.4 μg/mg, respectively), but higher catalase production did so (6). Therefore, the amphotericin B mechanisms of resistance in *Aspergillus* spp. need to be further elucidated.

In conclusion, we propose amphotericin B species-specific ECVs of 2 to 4 μg/ml for six *Aspergillus* spp. Our results cover amphotericin B and its lipid formulations, because MIC data have been comparable (18). Further studies are needed to determine the relationship between molecular mechanisms of resistance and our proposed amphotericin B non-WT values. Although ECVs do not predict clinical outcome of therapy, they should be considered for inclusion in future revised ver-

sions of the CLSI M38-A2 document. In the absence of clinical breakpoints, amphotericin B ECVs may aid in detecting isolates with reduced amphotericin B susceptibility (non-WT strains) harboring resistance mutations.

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