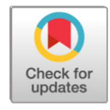

Mycology | Full-Length Text

Evaluation of the Vitek 2 system for antifungal susceptibility testing of *Candida auris* using a representative international panel of clinical isolates: overestimation of amphotericin B resistance and underestimation of fluconazole resistance

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ABSTRACT Although the Vitek 2 system is broadly used for antifungal susceptibility testing of *Candida* spp., its performance against *Candida auris* has been assessed using limited number of isolates recovered from restricted geographic areas. We therefore compared Vitek 2 system with the reference Clinical and Laboratory Standards Institute (CLSI) broth microdilution method using an international collection of 100 *C. auris* isolates belonging to different clades. The agreement ±1 twofold dilution between the two methods and the categorical agreement (CA) based on the Centers for Disease Control and Prevention's (CDC's) tentative resistance breakpoints and Vitek 2-specific wild-type upper limit values (WT-ULVs) were determined. The CLSI-Vitek 2 agreement was poor for 5-flucytosine (0%), fluconazole (16%), and amphotericin B (29%), and moderate for voriconazole (61%), micafungin (67%), and caspofungin (81%). Significant interpretation errors were recorded using the CDC breakpoints for amphotericin B (31% CA, 69% major errors; MaEs) and fluconazole (69% CA, 31% very major errors; VmEs), but not for echinocandins (99% CA, 1% MaEs for both micafungin and caspofungin) for which the Vitek 2 allowed correct categorization of echinocandin-resistant *FKS1* mutant isolates. Discrepancies were reduced when the Vitek 2 WT-ULV of 16 mg/L for amphotericin B (98% CA, 2% MaEs) and of 4 mg/L for fluconazole (96% CA, 1% MaEs, 3% VmEs) were used. In conclusion, the Vitek 2 system performed well for echinocandin susceptibility testing of *C .auris*. Resistance to fluconazole was underestimated whereas resistance to amphotericin B was overestimated using the CDC breakpoints of ≥32 and ≥2 mg/L, respectively. Vitek 2 minimun inhibitory concentrations (MICs) >4 mg/L indicated resistance to fluconazole and Vitek 2 MICs ≤16 mg/L indicated non-resistance to amphotericin B.

KEYWORDS *Candida auris*, Vitek 2, wild-type upper limit value, antifungal susceptibility testing, resistance

Candida auris is a rapidly emerging pathogenic yeast that has been associated with life-threatening invasive infections and numerous outbreaks in healthcare settings worldwide (1). Notably, it is the first and so far only fungal pathogen classified as an urgent health threat by the Centers for Disease Control and Prevention (CDC) as early as 2019 (2), while it is unsurprisingly listed in the critical priority group of fungi responsible for systemic infections recently declared by the World Health Organization (3). Unlike other *Candida* spp., *C. auris* is often resistant to different classes of antifungals (4–13), a feature particularly distressing that warrants enduring attention taking into account the limited therapeutic options. Therefore, antifungal susceptibility testing (AFST) can be

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considered a key parameter to guide therapy and to alert physicians to novel resistance patterns (14).

The Vitek 2 system (bioMérieux) is an automated approach for AFST of clinically significant yeasts, the use of which has become popular since it significantly diminishes hands-on and turnaround times as well as variability through a standard operating procedure. It constitutes a miniaturized version of the broth microdilution (BMD) method and its principle is based on repetitive turbidimetric monitoring of fungal growth during an abbreviated incubation period. Previous evaluations have revealed high levels of reproducibility and categorical agreement (CA; >92%) with the reference Clinical and Laboratory Standards Institute (CLSI) BMD method for common *Candida* spp. and most antifungal agents included in the Vitek 2 cards (15, 16). However, these findings cannot be safely generalized to all *Candida* spp. due to the unique growth characteristics and metabolic activity of each species. Notably, the performance of accurate AFST against *C. auris* has recently been caught in the spotlight of attention. Yet, although *C. auris* AFST with Vitek 2 system is not recommended by the manufacturer, comparative evaluations of the Vitek 2 and BMD methods for *C. auris* AFST show clade-dependent contradictory results and are based on assessments testing isolates recovered from restricted geographical areas (17–22).

Importantly, commercial AFST assays do not always mirror the reference methodology precisely resulting in incorrect classifications when the breakpoints (BPs) of the latter are applied. Previous studies have suggested that Vitek 2 susceptibility data would be more robust if either method- or species-specific epidemiological cutoff values (ECVs) would be available for each *Candida* spp. and antifungal drug evaluated, similarly to other commercial AFST assays, such as Sensititre YeastOne (23). Currently, the CDC has proposed tentative minimum inhibitory concentration (MIC) BPs for resistance to fluconazole, amphotericin B, and echinocandins for *C. auris* (24), while species-specific tentative ECVs have been suggested (25, 26). Still, no Vitek 2-specific interpretive criteria are available for *C. auris* and any antifungal agent.

Based on these grounds, we assessed the Vitek 2 system performance for *C. auris* AFST compared to the reference CLSI BMD method using an international collection of well-characterized clinical isolates selected to cover diverse susceptibility phenotypes in an attempt to evaluate Vitek 2 system in the global context and determine method-specific clade-dependent and -independent wild-type upper limit values (WT-ULVs) for MIC interpretation.

RESULTS

CLSI method

The modal (range) CLSI MICs for each clade separately and all isolates are shown in Table 1. The absolute inter-observer agreement for CLSI MICs obtained for all antifungals was excellent (96%), while the MIC values for the quality control strains were within the reference ranges.

The CLSI MIC distributions of *C. auris* isolates by clade are presented in Fig. 1. Overall, narrow unimodal MIC distributions were found for amphotericin B and 5-flucytosine (3–4 twofold dilutions), whereas wider MIC distributions were observed for echinocandins (4–7 twofold dilutions of non-*FKS* mutants) and azoles, mainly voriconazole (5–12 twofold dilutions). All isolates were interpreted as amphotericin B-non-resistant, regardless of clade, with clade I strains having a slightly higher modal MICs than the modal MIC of the other clades (1 versus 0.5 mg/L, respectively). All clade III strains were fluconazole-resistant (MIC >64 mg/L), whereas the fluconazole resistance rate of clade I, II, IV and V isolates was 83%, 67%, 77%, and 40%, respectively. All clade I isolates carrying mutations on *FKS1* were both micafungin- and caspofungin-resistant and displayed additional resistance to fluconazole. 5-Flucytosine modal MICs of clade I and III isolates were slightly lower than the modal MICs of strain from the other clades (0.06 versus 0.125–0.25 mg/L).

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TABLE 1 Clade-specific CLSI- and Vitek 2-obtained amphotericin B, fluconazole, and echinocandins' MIC data for *C. auris* isolates

Antifungal agent	Clade	Modal (range) MIC (mg/L)[a]		Median (range)	% Agreement		CDC R BP	% CA (MaE, VmE)	Vitek 2 WT-	% CA (MaE, VmE) based
	(*N*)	CLSI	Vitek 2	difference[b]	±1	±2	(mg/L)	based on CDC BP	ULV (mg/L)	on Vitek 2 WT-ULV
Amphotericin B	All (100)	0.5 (0.25–1)	8 (≤0.25–>16)	3 (0 to 5)	29%	40%	2	31% (69%, 0%)	16	98% (2%, 0%)
	I (47)	1 (0.25–1)	8 (0.5–>16)	3 (0 to 5)	6%	19%	2	6% (94%, 0%)	16	96% (4%, 0%)
	II (3)	0.25 (0.25–0.5)	0.5 (≤0.25–0.5)	0 (0 to 1)	100%	100%	2	100% (0%, 0%)	2[c,d]	100% (0%, 0%)
	III (23)	0.5 (0.25–0.5)	0.5 (0.5–2)	1 (0 to 2)	87%	100%	2	96% (4%, 0%)	2[d]	100% (0%, 0%)
	IV (22)	0.5 (0.5–1)	8 (1–8)	4 (1 to 4)	5%	5%	2	4% (96%, 0%)	16	100% (0%, 0%)
	V (5)	0.5 (0.5–1)	1/4 (1–4)	2 (1 to 3)	40%	80%	2	40% (60%, 0%)	16[c]	100% (0%, 0%)
Fluconazole	All (100)	>64 (4–>64)	32 (≤0.5–>32)	−2 (−6 to 1)	16%	78%	32	69% (0%, 31%)	4	96% (1%, 3%)
	I (47)	>64 (4–>64)	16 (≤0.5–>32)	−2 (−6 to 1)	26%	79%	32	53% (0%, 47%)	4	98% (0%, 2%)
	II (3)	>64 (4–>64)	32 (2–32)	−2 (−2 to −1)	33%	100%	32	100% (0%, 0%)	4[c]	100% (0%, 0%)
	III (23)	>64 (>64–>64)	32 (8–32)	−2 (−4 to −2)	0%	96%	32	96% (0%, 4%)	4	100% (0%, 0%)
	IV (22)	>64 (8–>64)	32 (1–32)	−2 (−6 to −2)	0%	55%	32	64% (0%, 36%)	4	91% (0%, 9%)
	V (5)	4/>64 (4–>64)	2 (2–>32)	−1 (−3 to 1)	60%	80%	32	100% (0%, 0%)	8[c,e]	100% (0%, 0%)
Micafungin	All (100)	0.03 (0.016–>8)	0.125 (≤0.06–>4)	1 (−2 to 3)	67%	97%	4	99% (1%, 0%)	1	99% (1%, 0%)
	I (47)	0.03 (0.016– >8)	0.125 (≤0.06–>4)	1 (−2 to 3)	70%	96%	4	98% (2%, 0%)	1	98% (2%, 0%)
	II (3)	0.03 (0.03–0.03)	≤0.06 (≤0.06–≤0.06)	1 (1 to 1)	100%	100%	4	100% (0%, 0%)	ND (1)[f]	100% (0%, 0%)
	III (23)	0.03 (0.03–0.06)	0.125 (0.125–0.25)	2 (1 to 3)	30%	96%	4	100% (0%, 0%)	1	100% (0%, 0%)
	IV (22)	0.06 (0.03–0.06)	0.125 (≤0.06–0.125)	1 (0 to 2)	91%	100%	4	100% (0%, 0%)	ND (1)[f]	100% (0%, 0%)
	V (5)	0.03 (0.03–0.06)	≤0.06 (≤0.06–0.25)	1 (1 to 2)	80%	100%	4	100% (0%, 0%)	0.5[c, g]	100% (0%, 0%)
Caspofungin	All (100)	0.5 (0.125– >8)	0.25 (≤0.125–>4)	−1 (−2 to 2)	81%	100%	2	99% (1%, 0%)	2	99% (1%, 0%)
	I (47)	0.5 (0.125– >8)	0.25 (≤0.125–>4)	−1 (−2 to 2)	72%	100%	2	98% (2%, 0%)	2	98% (2%, 0%)
	II (3)	0.5 (0.5–0.5)	≤0.125 (≤0.125–≤0.125)	−2 (−2 to −2)	0%	100%	2	100% (0%, 0%)	ND (2)[f]	100% (0%, 0%)
	III(23)	0.5 (0.25–1)	0.25 (0.25–0.5)	−1 (−2 to 0)	96%	100%	2	100% (0%, 0%)	2	100% (0%, 0%)
	IV (22)	0.5 (0.25–0.5)	0.25 (0.25–0.25)	−1 (−1 to 0)	100%	100%	2	100% (0%, 0%)	ND (2)[f]	100% (0%, 0%)
	V (5)	0.25/0.5 (0.25–1)	≤0.125/0.25 (≤0.125–0.5)	−1 (−2 to 0)	60%	100%	2	100% (0%, 0%)	2[c]	100% (0%, 0%)

[a]For bimodal distributions, both modal MICs are presented.
[b]Number of twofold dilutions.
[c]The WT-ULV should be interpreted cautiously because of the low number of isolates tested.
[d]For both clades II and III, a WT-ULV of 16 mg/L would result in 100% CA (0% MaE, 0% VmE).
[e]A WT-ULV of 4 mg/L would result in 80% CA (0% MaE, 20% VmE).
[f]For clade II where the MICs were off-scale and clade IV where most of the MICs were the same, a WT-ULV could not be determined (ND), and thus, the WT-ULV of all isolates was used and it is presented in brackets.
[g]A WT-ULV of 1 mg/L would result in 100% CA (0% MaE, 0% VmE).

Vitek 2 system

The Vitek 2 MIC results were obtained after 12.2 h to 18.7 h of incubation (average time to reading, 14.6 h). The modal (range) Vitek 2 MICs for each clade separately and all isolates are shown in Table 1. The median (range) difference between replicates of Vitek 2 MICs for all antifungals was 0 (−2 to 2) twofold dilutions corresponding to an absolute/±1 $\log_2$ dilution inter-experimental method's agreement of 77%/90%. Notably, the CA between the independent replicates was excellent for micafungin (100%), caspofungin (100%) and amphotericin B (95%), but not for fluconazole (70%). The Vitek 2 MIC values for the quality control isolates were within the expected ranges.

The Vitek 2 MIC distributions of *C. auris* isolates by clade are depicted in Fig. 2. Clade-specific amphotericin B MIC distributions were found, with clade II and III isolates having lower MICs (modal MIC 0.5 mg/L) than clade I and IV isolates (modal MIC 8 mg/L), and clade V strains having in-between MICs (1–4 mg/L). Thus, all clade II and 96% of clade III isolates were interpreted as amphotericin B-non-resistant (MIC ≤0.5 mg/L), while the amphotericin B resistance rate of clade I, IV, and V isolates was 94%, 95%, and 60%, respectively.

Concerning the azoles, the MIC ranges were wide spanning eight twofold dilutions and clade-specific differences were observed. Fluconazole resistance rates varied from 36%, 41%, and 40% for clade I, IV, and V isolates to 66% and 96% for clade II and III isolates, respectively. Clade-specific voriconazole MIC distributions were found, with

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FIG 1 CLSI MIC distributions of *C. auris* isolates by clade. The broken lines indicate the CDC's tentative BPs for *C. auris* (where available) (24).

clade I isolates having lower MICs than clade III isolates (modal MIC ≤0.125 versus 2 mg/L, respectively).

With regards to echinocandins, the MIC ranges were narrow (four twofold dilutions excluding the *FKS1* mutants). All clade I *FKS1* mutant strains were both micafungin- and caspofungin-resistant and demonstrated additional resistance to amphotericin B (9/9; 100%), while a proportion (4/9; 44%) did not exhibit resistance to fluconazole.

Finally, most (94/100) isolates had low off-scale 5-flucytosine MICs (modal MIC ≤1 mg/L).

CLSI method versus Vitek 2 system

Amphotericin B

The CLSI-Vitek 2 agreement within ±1 twofold dilution was poor for amphotericin B (29%), with a median (range) difference of 3 (0 to 5) twofold dilutions. Slightly higher agreement was found within ±2 twofold dilutions (40%). Although the MIC values generated by the two methods were significantly different ($P < 0.0001$), they demonstrated significant correlation (Pearson r, 95% CI 0.64, 0.51–0.74, $P < 0.0001$) indicating that the Vitek 2 MIC distribution is shifted by an average three twofold dilutions higher than the CLSI MIC distribution. Albeit all isolates were interpreted as non-resistant based on the CLSI, 69/100 strains were resistant to amphotericin B according to the Vitek 2 corresponding to 31% CA [Cohen's kappa coefficient (κ) = 0; no agreement and 69% major errors (MaEs) (Fig. 3).

As the Vitek 2 amphotericin B MIC distribution was bimodal with different MIC distributions depending on the clade, two clade-specific WT-ULVs were determined with the ECOFFinder, namely 2 mg/L for clade II and III isolates and 16 mg/L for clade I, IV, and V isolates. Based on these WT-ULVs, discrepancies were overall minimized to 98% CA (2% MaEs). However, as clade information may not be available at the time of AFST, one could use the WT-ULV of 16 mg/L regardless of clade with the caveat to lose some clade II and III non-WT isolates with MICs of 4–16 mg/L (Table 1).

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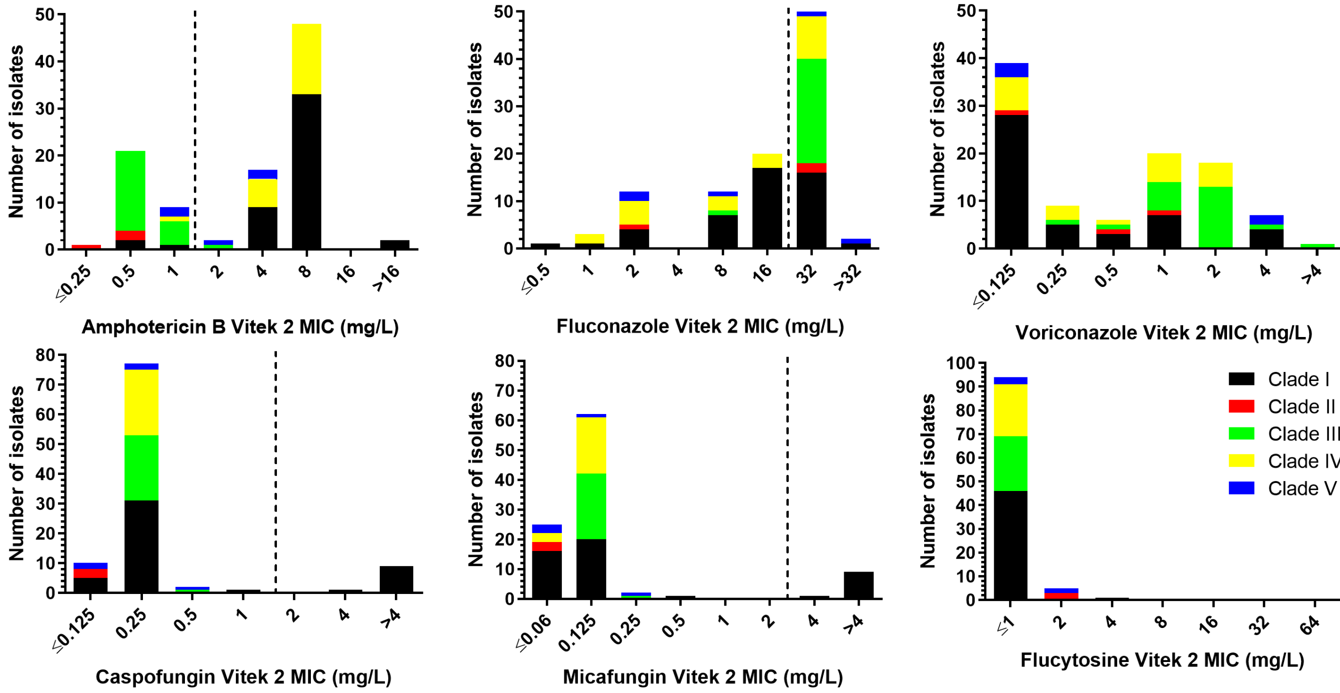

FIG 2 Vitek 2 MIC distributions of *C. auris* isolates by clade. The broken lines indicate the CDC's tentative BPs for *C. auris* (where available) (24).

Fluconazole

Similarly, the CLSI-Vitek 2 agreement within ±1 $\log_2$ dilution was poor for fluconazole (16%), with a median (range) difference of −2 (−6 to 1) twofold dilutions. Greater agreement was found within ±2 $\log_2$ dilutions (78%). Although the MIC values obtained by the two methods were significantly different ($P < 0.0001$), they showed strong correlation (Pearson *r*, 95% CI 0.73, 0.62–0.81, $P < 0.0001$) indicating that the Vitek 2 MIC distribution is shifted by an average of two twofold dilutions lower than the CLSI MIC distribution. Notable interpretation discrepancies were recorded since 83/100 isolates were interpreted as resistant as per the CLSI MICs, while only 52/100 strains exhibited fluconazole resistance based on the Vitek 2 corresponding to 69% CA ($\kappa = 0.36$, 95% CI 0.22–0.51; fair agreement), no MaEs and 31% very major errors (VmEs) (Fig. 3).

Of note, Vitek 2 false non-resistant results occurred for isolates belonging to specific clades. In particular, none of the fluconazole-resistant clade II and V isolates were falsely categorized as fluconazole-non-resistant based on the CDC's tentative resistance BP of 32 mg/L (24), as opposed to 22/39 (56% VmEs), 1/23 (4% VmEs), and 8/17 (47% VmEs) fluconazole-resistant clade I, III, and IV isolates, respectively, which were incorrectly interpreted as fluconazole-non-resistant (Fig. 3). Nevertheless, using a Vitek 2-specific WT-ULV of 4 mg/L regardless of clade, the CA increased to 96% (1% MaEs, 3% VmEs). Notably, the CA for clade V isolates will be higher using a WT-ULV of 8 mg/L (100% versus 80%) (Table 1).

Echinocandins

On the other hand, the CLSI-Vitek 2 agreement within ±1 twofold dilution was moderate for micafungin (67%) and caspofungin (81%), with a median (range) difference of 1 (−2 to 3) and −1 (−2 to 2) twofold dilutions, respectively. Excellent agreement was found within ±2 twofold dilutions (97% for micafungin and 100% for caspofungin). The MIC values for both echinocandins generated by the two methods did not differ significantly ($P > 0.7$) and showed moderate correlation (Pearson *r*, 95% CI 0.61, 0.37–0.74 for micafungin and 0.62, 0.48–0.73 for caspofungin, $P < 0.0001$). All but one of the echinocandin-non-resistant isolates were correctly classified as such with the Vitek 2

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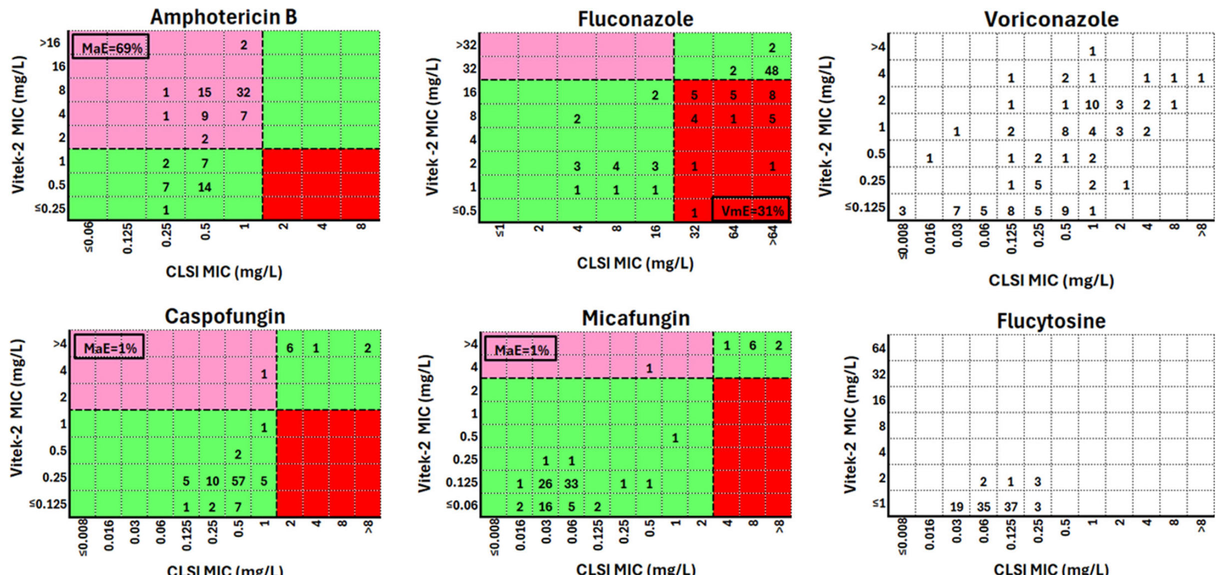

FIG 3 Scatter plots of CLSI MICs versus Vitek 2 MICs. Numbers represent the number of *C. auris* isolates (total n = 100) at each MIC pair. The black broken lines indicate the CDC's tentative BPs for *C. auris* (where available) (24). The green shaded areas represent CA, while the pink and red areas indicate MaE and very major error (VmE), respectively.

system corresponding to 99% CA (κ = 0.94, 95% CI 0.83–1; almost perfect agreement), 1% MaEs and no VmEs, for both micafungin and caspofungin (Fig. 3). For micafungin, Vitek 2 MICs retrieved using *C. albicans* and *C. glabrata* as the species for AFST differed for 32/100 isolates, with one isolate having a MIC of 0.25 mg/L and 31 isolates having a MIC of 0.125 mg/L when *C. albicans* was used, and a MIC of ≤0.06 mg/L for all 32 isolates when *C. glabrata* was used, without significantly changing the comparison with the CLSI method (99% CA; 1% MaEs and 0% VmEs). Similarly, for caspofungin, different MICs were found for 16/100 strains with 15 isolates having a MIC of 0.25 mg/L and one isolate having a MIC of 1 mg/L when *C. albicans* was used, and 0.5 and 4 mg/L, respectively, when *C. glabrata* was used, without significantly changing the comparisons with the CLSI method (98% CA; 2% MaEs and 0% VmEs).

The estimated Vitek 2-specific WT-ULV encompassing >99% of isolates was 1 mg/L for micafungin and 2 mg/L for caspofungin resulting in a 99% CA (one false-resistant isolate). When clade-specific Vitek 2 WT-ULVs were determined, a cutoff micafungin/caspofungin MIC value of 1/2 mg/L, 1/2 mg/L, and 0.5/2 mg/L was found for clade I, clade III, and clade V isolates, respectively. The echinocandins' Vitek 2 MICs of clade II strains were off-scale and most of the MICs of clade IV strains were the same, and thus they were not acceptable for WT-ULV estimation (Table 1).

5-Flucytosine and voriconazole

With regard to 5-flucytosine, the CLSI-Vitek 2 agreement within ±1/±2 $\log_2$ dilutions was poor (0%/3%), with a median (range) difference of ≥4 (≥2 to ≥5) twofold dilutions. The MIC values obtained with the two methods were significantly different (P < 0.0001) and weakly correlated (Pearson r, 95% CI 0.35, 0.16–0.52, P = 0.0004), although the vast majority (94%) of the isolates had off-scale Vitek 2 MICs. On the contrary, the CLSI-Vitek 2 agreement within ±1/±2 $\log_2$ dilutions was moderate (61%/86%) for voriconazole, with a median (range) difference of 0 (−3 to 5). The MIC values obtained by the two methods did not differ significantly (P = 0.97) and showed moderate correlation (Pearson r, 95% CI 0.44, 0.26–0.58, P < 0.0001), although 40% of the isolates had off-scale Vitek 2 MICs (Fig. 3).

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Vitek 2 5-flucytosine and voriconazole MIC distributions were truncated at the lower end within the likely WT population, and thus were not acceptable for WT-ULV estimation (Fig. 2).

DISCUSSION

Timely administration of appropriate antifungal therapy based on accurate AFST data is considered a cornerstone in the management of fungal infections. Herein, the comparative evaluation of the Vitek 2 system and the CLSI reference BMD method for AFST against a global collection of *C. auris* isolates showed that the automated system demonstrated clade- as well as drug-related performance patterns. Overall, the Vitek 2-derived amphotericin B MICs were significantly higher (29% agreement within ±1 twofold dilution) than the CLSI amphotericin B MICs leading to considerable interpretation errors (69% MaEs, 0% VmEs), which were limited when a Vitek 2-specific WT-ULV of 16 mg/L was used (2% MaEs, 0% VmEs). With regard to fluconazole, the Vitek 2-derived MICs were significantly lower (16% agreement within ±1 twofold dilution) than the CLSI MICs resulting in notable interpretation discrepancies (0% MaEs, 31% VmEs), which were restricted when a Vitek 2-specific WT-ULV of 4 mg/L was applied (1% MaEs, 3% VmEs). On the contrary, the echinocandins MIC values obtained by the two methods did not differ significantly (67% and 81% agreement within ±1 twofold dilution for micafungin and caspofungin, respectively) and the Vitek 2 reliably classified all *FKS* hotspot mutant strains as echinocandin-resistant (1% MaEs, 0% VmEs). The Vitek 2-CLSI agreement within ±1 twofold dilution was moderate for voriconazole (61%) and poor for 5-flucytosine (0%).

While the Vitek 2 system is broadly used in routine clinical laboratories, comparative evaluations with the CLSI method for AFST against *C. auris* rely on testing of isolates recovered from restricted geographical areas, i.e., belonging to specific clades (17–22). To date, studies on *C. auris* antifungal susceptibility profiles, which have mainly been carried out using the reference BMD methodology, indicate that MIC distributions can vary significantly among isolates from different clades (27). Indicatively, when AFST was performed based on the CLSI BMD method, 62%, 0%, and 0% of clade II isolates from Korea have been reported to be resistant to fluconazole, amphotericin B, and echinocandins, respectively (17), whereas the corresponding rates for clade I isolates from India were 86%–90%, 8%–10%, and 2%–6% (25, 28), and for clade IV isolates from Colombia, rates were 94%, 0%, and 0% (18). As resistance was not always confirmed moleualry, such variations may be attributed to differences in the cell morphology and structure, particularly the capacity to produce aggregates (29), as well as the unique metabolic properties (30) of each clade-specific *C. auris* phenotype.

Considering the aforementioned variable regional patterns of antifungal resistance, clade-dependent conflicting Vitek 2 performance characteristics for *C. auris* AFST compared to the CLSI reference method have been reported, precluding their safe extrapolation in the general context. In particular, Kwon et al. showed that the Vitek 2 system can be a reliable tool for fluconazole AFST against clade II isolates (97% agreement within ±2 twofold dilutions, 93% CA) (17). On the contrary, Ceballos-Garzon et al. reported that the Vitek 2 fluconazole data for clade IV strains should be interpreted with caution (61% CA, 39% VmEs) (18). A possible explanation for this notable difference could be the composition of the CLSI-based fluconazole-resistant population within the collection of isolates tested (62% for clade II versus 96% for clade IV). In fact, when studies are performed using an abundance of susceptible isolates, VmEs are uncommon, as opposed to studies conducted testing an abundance of resistant strains (31, 32). Similar clade-dependent contradictory results were found for amphotericin B. The Vitek 2 system showed excellent performance for amphotericin B AFST against clade II isolates (100% agreement within ±2 twofold dilutions, 100% CA) (17, 22), although that was not the case for testing clade I (15%–16% CA, 84%–85% MaEs) (20, 21) as well as clade IV (10% CA, 90% MaEs) (18) strains. It can be postulated that clade-specific differences in the CLSI amphotericin B MIC distributions are responsible for such a variation, since the

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modal MIC for clade I (21) and clade IV (18) isolates is just one and two twofold dilution step lower from the CDC's tentative BP of 2 mg/L (24), as opposed to the corresponding value for clade II strains (0.5 mg/L) (22).

To our knowledge, this is the first assessment of the Vitek 2 performance for *C. auris* AFST encompassing well-characterized, genetically distinct *C. auris* isolates from diverse geographical origins covering all five clades. This selection criterion also restricted the risk of overrepresentation of clonal strains that could affect the present evaluation as well as future comprehensive performance comparisons of the commercial assay. Of note, isolates were genotyped only in one (17) of the previous published studies (18–22), and thus low genetic variability cannot be excluded. Moreover, we included truly echinocandin-resistant (*FKS1*-mutation containing) strains, in contrast to all previous comparative evaluations (17–22). This is of particular importance given that echinocandins are currently indicated as first-line therapy for *C. auris* infections (33). Nevertheless, an ever-expanding number of breakthrough *C. auris* diseases, mainly catheter-related, associated with *FKS1* mutant isolates have recently been reported (4, 6, 8–10, 12). Consequently, accurate echinocandin AFST data of *C. auris* are a significant component of a potentially effective therapy design, and thus, it is crucial to test a panel of resistant as well as non-resistant strains, in order to assess reliable the performance of a susceptibility assay.

Our CLSI micafungin susceptibility data are consistent with previous reports (modal MIC and MIC_{90} values of 0.03 and 0.5 mg/L in the present study versus 0.03–0.125 mg/L and 0.25–0.5 mg/L, respectively) (18, 19, 28), with the majority of MIC values being gathered four twofold dilution steps lower than the CDC's tentative BP of 4 mg/L (24). The clade I isolates bearing S639F or ΔF635 mutations showed higher CLSI micafungin MIC values (4–>8 mg/L) compared with the WT isolates, as previously described (28). The Vitek 2 micafungin MICs were one to two twofold dilutions higher than the CLSI MICs, i.e., close to the CDC's BP, resulting in an almost perfect CA of 99% (1% MaEs, 0% VmEs), which is in agreement with previous studies (17–20). While the level of previously reported CA (100%) (17–20) would not change adopting the Vitek 2-specific micafungin WT-ULV of 1 mg/L proposed here, which is close to the suggested CLSI ECV of 0.5 mg/L (25, 26), further verification assessments incorporating *FKS* hotspot mutant strains with MICs one to three twofold dilutions higher than the ECV are warranted in order to elucidate whether a BP of 4 mg/L or a BP closer to the ECV predicts clinical outcome.

With regard to caspofungin, one should keep in mind that its role as a surrogate marker for echinocandin resistance is questionable due to the marked inter-laboratory variability that has been observed among the BMD-derived caspofungin MICs for *Candida* spp. (34). Specifically for *C. auris*, BMD susceptibility testing to caspofungin has been discouraged since caspofungin induces significant Eagle effect resulting in an overestimation of the resistant population, while only *FKS* mutant isolates are truly echinocandin-resistant (35). Of note, the Vitek 2 system reliably classified all our *C. auris* strains harboring *FKS* hotspot mutations as caspofungin-resistant based on the CDC's tentative BP of 2 mg/L (24). Nevertheless, the previously reported 37% caspofungin resistance rate in *C. auris*, which was determined by both the CLSI and the Vitek 2, but without supporting molecular data (sequencing of *FKS* hotspot regions using known *FKS C. glabrata* primers due to lack of published genomic data for *C. auris* at that time) (21), could be attributed to the propensity of the isolates to exhibit a paradoxical growth effect. Hence, if *FKS* sequence analysis is not available on a laboratory routine basis, the Vitek 2 micafungin MIC values should be used as an indicator of resistance to echinocandins.

Given that treatment with liposomal amphotericin B could be considered as an alternative therapeutic option for *C. auris* infections (33), an issue of significant concern is the misleading elevated Vitek 2 amphotericin B MICs found for most of the isolates tested in the present study (agreement within ±1/±2 $\log_2$ dilutions 29%/40%) resulting in 31% CA (69% MaEs), which is in line with other reports (18, 20, 21). Only clade II strains were not falsely categorized as amphotericin B-resistant based on the Vitek 2

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(100% CA), as previously described (17, 22). Our CLSI amphotericin B susceptibility data are consistent with those of previous reports (modal MIC and MIC_{90} values of 0.5–1 mg/L versus 1–2 mg/L, respectively) (17, 18, 28, 29), with the majority (88%) of the MIC values being tightly clustered within one to two twofold dilutions lower from the CDC's tentative resistance BP of 2 mg/L (24). Isolates with known resistance mechanisms were not included for the CLSI-Vitek 2 comparison. Notably, mutations within the *ERG3*, *ERG5*, *ERG6*, and/or *ERG10* genes, which have been associated with amphotericin B resistance in other *Candida* spp., have not been previously identified among phenotypically amphotericin B-resistant *C. auris* strains, and thus, resistance could not be verified molecularly (9, 36, 37). In fact, the first mechanisms contributing to clinical amphotericin B resistance in *C. auris*, conferred by mutations in the *ERG6* (13) and the *ERG3* (4) genes, have only been recently identified. Nevertheless, there are indications that amphotericin B resistance in *C. auris* is inducible and transient, with the MIC values of some strains being reduced following passage in the laboratory (38).

Regardless of clade, unimodal CLSI amphotericin B MIC distributions for *C. auris* have been described (17, 18, 28, 29), corroborating our findings and indicating a relatively low level of resistance, which is further endorsed by a recent meta-analysis in the global epidemiology of *C. auris* showing an overall amphotericin B resistance rate of 12% (39). Nonetheless, an amphotericin B resistance rate of 94% (modal MIC and MIC_{90} values of 8 and >16 mg/L, respectively) as per the CDC's tentative BP of 2 mg/L (24) was reported, when the susceptibility profile of 48 Colombian *C. auris* isolates was determined by Vitek 2 (18, 40). Interestingly, when the MICs were re-assessed using gradient concentration strips (Etest), the rate of amphotericin B-resistant strains decreased to 15% (modal MIC and MIC_{90} values of 1 and 2 mg/L, respectively) (18, 40). The corresponding results obtained for 90 Indian *C. auris* isolates were more pronounced, since amphotericin B resistance rate was 100% (modal MIC and MIC_{90} values of 8 and >16 mg/L, respectively) based on Vitek 2-derived MICs and just 1% (modal MIC and MIC_{90} values of 0.5 and 0.5 mg/L, respectively) according to Etest-generated susceptibility data (21). Worryingly, overestimation of amphotericin B resistance in *C. auris* has also been demonstrated with another widely used BMD-based AFST assay (Sensititre YeastOne) (41). These caveats underline the remarkable variability in amphotericin B MIC values for *C. auris* across different testing methodologies, as has been recently supported by the CLSI (42), and call for definition of method-specific ECVs. Of note, the resistance rate would be reduced to 8% and 0% for the aforementioned Colombian and Indian *C. auris* strains, respectively, adopting the Vitek 2-specific amphotericin B WT-ULV of 16 mg/L proposed here.

While most (91%) *C. auris* isolates are resistant to fluconazole (39), there are fluconazole-non-resistant strains, particularly within the clade II and to a lesser extent within the clades I and IV (38), which is in agreement with our clade-specific CLSI fluconazole distributions. Although persistent or breakthrough *C. auris* infections caused by fluconazole-non-resistant isolates (MICs 2–8 mg/L) have been reported (43) questioning the CDC's tentative BP of 32 mg/L (24), the critical function of an AFST assay is to provide a high rate of accurate and reproducible results. Based on our findings, neither of these criteria was fulfilled for Vitek 2 fluconazole MIC testing against *C. auris*. Overall, the Vitek 2 fluconazole MICs were two twofold dilutions lower than the CLSI MICs (agreement within ±1/±2 $\log_2$ dilutions 16%/78%) leading to 69% CA (0% MaEs, 31% VmEs), as previously described (18–20). Furthermore, categorical disagreement of 30% between the independent replicates tested was recorded.

Of note, the Vitek 2 interpretations are both species- and drug-specific, while the susceptibility panel is not yet validated for *C. auris* AFST. Nevertheless, it is not unusual for laboratories to exploit the cards' microdilutions by changing the species definition to a valid one so as to allow for MICs report in cases that treatment decisions are needed urgently. Intriguingly, Korem et al. showed that a modified AST-YS08 card to assess the adequacy of fluconazole MICs for *C. glabrata* by setting the species name to *C. albicans* within the Vitek 2 software produced 12% VmEs (44). Hence, laboratories should be aware that species modification may not be an accurate approach for *C.*

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auris susceptibility testing to fluconazole. However, an alternative approach to detect fluconazole-resistant *C. auris* isolates could be the adoption of the Vitek 2-specific fluconazole WT-ULV of 4 mg/L proposed here as it would increase the CA to 96% (1% MaEs, 3% VmEs).

Although fluconazole-resistant *C. auris* isolates may respond to other triazoles occasionally, the use of fluconazole susceptibility profile is currently suggested as a marker for second-generation triazole susceptibility assessments (24). Our CLSI voriconazole MIC data are comparable with those previously reported demonstrating wide MIC distributions (spanned 5 to 12 twofold dilutions) regardless of clade (17, 18, 21, 29, 45). The Vitek 2 voriconazole MIC values did not differ significantly from the corresponding CLSI MICs (agreement within ±1/±2 $\log_2$ dilutions 61%/86%), which is in agreement with other reports (17, 19). It is very likely that the wide MIC range is due to multiple resistance mechanisms accumulated by *C. auris* isolates, and therefore, most of the strains should be considered resistant since voriconazole CLSI MICs were correlated with fluconazole CLSI MICs (Pearson *r*, 95% CI 0.62, 0.48–0.73, $P < 0.0001$). Considering fluconazole as a surrogate marker of azole resistance, a voriconazole WT-ULV of 0.06 mg/L would result in CA of 90% between the two drugs with 6% MaE and 4% VmE (data not shown) rendering most isolates resistant to voriconazole as to fluconazole. A WT-ULV of 0.06 mg/L is lower than the lowest end of the card's calling range (0.125 mg/L) (46), which, in combination with the off-scale MICs, precludes the estimation of CA with the CLSI.

With regard to 5-flucytosine, our CLSI susceptibility data are in line with those previously described (modal MIC, MIC_{50}, and MIC_{90} values of 0.125, 0.06, and 0.125 mg/L versus 0.06–0.125 mg/L, 0.06–0.125 mg/L, and 0.25–64 mg/L, respectively) (19, 28, 29). The CLSI-Vitek 2 agreement was poor (0% and 3% within ±1 and ±2 twofold dilutions, respectively) since the vast majority (94%) of the Vitek 2 MIC values were off-scale at the lower end of the card's calling range, as previously described (19). Given that 1 mg/L is the lowest 5-flucytosine concentration contained in the Vitek 2 AST-YS08 card (46), its concentration range should be extended to enable further optimization of *C. auris* AFST.

Taken together, *C. auris* may become a driving force in the widespread application of AFST owing to its elevated levels of documented resistance, which may be characterized by regional patterns. The Vitek 2 allowed correct categorization of all echinocandin-resistant *FKS1* mutant isolates. On the contrary, the non-resistance of *C. auris* to fluconazole and amphotericin B should be interpreted with caution as per the CDC's tentative BP of 32 and 2 mg/L, respectively (24), if the automated system is used to guide therapeutic decisions. Laboratories should bear in mind that *C. auris* isolates with amphotericin B MIC ≤16 mg/L may suggest non-resistance, whereas fluconazole MIC >4 mg/L may indicate resistance by Vitek 2 system. Further multicenter evaluations are required to corroborate the current findings and to determine Vitek 2-specific ECVs in order optimize the interpretation of the Vitek 2-derived MIC data.

MATERIALS AND METHODS

Isolates

A total of 100 clinical isolates were tested. Namely, 17 bloodstream isolates were collected from individual patients hospitalized in eight Greek tertiary care hospitals located in the Attica region from November 2020 to August 2022. They were identified to the species level using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Bruker Daltonics, Bremen, Germany) and clustered in clade I (South Asian) (47). In addition, 83 genetically distinct isolates belonging to all five *C. auris* clades and being isolated from various geographical regions were included. In particular, the aforementioned collection comprised of 30 strains from clade I (South Asian; Brazil, Kuwait, Iran, India, Oman, Pakistan), three strains from clade II (East Asian; South Korea, Japan), 23 strains from clade III (African; South Africa, Spain), 22 strains from clade IV (South American; Venezuela, Colombia), and five strains from clade V (Iranian; Iran) (47)

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all with different short tandem repeat profiles. The strain set included nine clade I *FKS1* hotspot mutant isolates harboring non-synonymous or deletion mutations (S639F and ΔF635) that confer resistance to echinocandins, detected as previously described (10).

All isolates were stored at −70°C in normal sterile saline with 10% glycerol (AppliChem, Darmstadt, Germany) until use. Prior to testing, they were revived by subculturing them twice onto in-house prepared antimicrobial-free Sabouraud dextrose agar (Oxoid, Athens, Greece) plates at 35±2°C for 24 h.

Antifungal susceptibility testing

CLSI method

The CLSI AFST was performed according to the M27A4 protocol guidelines using laboratory-grade pure powders of amphotericin B (Sigma-Aldrich, Athens, Greece), fluconazole (Sigma-Aldrich, Athens, Greece), voriconazole (Pfizer Ltd., Kent, UK), micafungin (Astellas Pharma, Tokyo, Japan), caspofungin (Merck & Co., NJ, USA) and 5-flucytosine (Sigma-Aldrich, Athens, Greece). Briefly, twofold serial drug concentrations ranging from 8 to 0.06 mg/L for amphotericin B, 64 to 1 mg/L for fluconazole, and 8 to 0.008 mg/L for the remaining antifungals were used. Inoculum suspensions were prepared in sterile water and were adjusted to the required concentration. The microtiter plates were incubated at 35±2°C and the MICs were evaluated by visual inspection of the plates with the aid of a magnifying mirror by two blinded observers after 24 h. Discordance in the BMD MICs was arbitrated by a third reader. The amphotericin B MICs were defined as the lowest drug concentration at which total inhibition of visual growth compared to the growth control well was observed, while the MICs of the rest of antifungals tested were read as the lowest drug concentration to produce a prominent decrease in turbidity (~50% visual growth reduction) relative to the growth control well (48).

Vitek 2 system

The Vitek 2 AFST was performed using the AST-YS08 cards (bioMérieux, Marcy l'Etoile, France) in accordance with the manufacturer's recommendations. The calling MIC range was 0.06 to 8 mg/L for micafungin, 0.125 to 8 mg/L for voriconazole and caspofungin, 0.25 to 16 mg/L for amphotericin B, 0.5 to 64 mg/L for fluconazole, and 1 to 64 mg/L for 5-flucytosine. Inoculum suspensions were prepared in 0.45% saline solution (bioMérieux, Marcy l'Etoile, France) and were adjusted to the desired concentration. The loaded cards were incubated for a maximum of 24 h in the Vitek 2 instrument and were read automatically. As the Vitek 2 system does not yet provide species-specific MICs for *C. auris*, the species was modified to different *Candida* spp. to retrieve the MICs for *C. auris* isolates. Of note, among the five species most commonly associated with candidiasis, i.e., *C. albicans*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, and *C. krusei*, the MIC values remained unchanged regardless of the species selected to retrieve the MIC data for *C. auris* for all drugs, except for echinocandins when *C. glabrata* was selected. Since AST-YS08 is not accredited by the United States Food and Drug Administration for fluconazole and caspofungin AFST against *C. glabrata* (46), analysis was based on MICs retrieved using *C. albicans*. In order to assess the potential variation in MIC determination by Vitek 2, a proportion of isolates (15/100) have been re-tested on different days so as to determine the method's inter-day reproducibility. Testing was repeated by both the CLSI BMD and Vitek 2 for the isolates displaying discordant results and the repeat result was kept as final.

All isolates were tested by both methods at the same laboratory on the same day using the same subculture plate. Inoculum density and purity checks were performed on all isolates by spread plate counts on in-house prepared antimicrobial-free Sabouraud dextrose agar plates. The recommended *C. krusei* ATCC 6258 and *C. parapsilosis* ATCC 22019 were used as quality control strains for both AFST methods.

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Analysis

A head-to-head comparison of the generated MIC data sets, using the CLSI BMD as the reference methodology, was performed. High off-scale MIC results were converted to the next highest twofold concentration, while low off-scale MIC values were left unchanged. For the quantitative analysis, the results of the two AFST methods were analyzed with paired Student's *t*-test after $\log_2$ transformation of the MIC data sets. A two-tailed *P*-value of <0.05 was considered to reveal a statistically significant difference. The levels of CLSI-Vitek 2 agreement within ±1 and ±2 twofold dilutions were calculated. For the qualitative analysis, the CA was estimated following the CDC's tentative resistance BPs for *C. auris* (where available), i.e., amphotericin B ≥2 mg/L, fluconazole ≥32 mg/L, micafungin ≥4 mg/L, and caspofungin ≥2 mg/L (24), and its strength was assessed by calculating the κ statistic. Discrepancies were considered as MaE when the CLSI classified an isolate as non-resistant and the Vitek 2 as resistant (false resistance), and VmE when the CLSI categorized a strain as resistant and the Vitek 2 as non-resistant (false non-resistance). Furthermore, CA was estimated based on the Vitek 2 WT-ULV determined for each antifungal using the ECOFFinder program (49). The WT-ULV is defined as the upper MIC value where the WT distribution ends, and it was used to classify isolates as WT with MICs ≤ WT ULV and non-WT with MIC > WT ULV. Since for the ECV determination the dataset needs to include at least five MIC distributions (at least 15 isolates/distribution) generated from separate centers and at least 100 MICs in the putative WT distribution, we abstain from using the term ECV but the WT-ULV which describes the WT population in the collection of the isolates used in the present study. All data were analyzed using the statistics software package GraphPad Prism, version 8.0, for Windows (GraphPad Software, San Diego, CA, USA).

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