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SUSCEPTIBILITY

Comparison of the MICs Obtained by Gradient Concentration Strip and EUCAST Methods for Four Azole Drugs and Amphotericin B against Azole-Susceptible and -Resistant *Aspergillus* Section *Fumigati* Clinical Isolates

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ABSTRACT Reference methods used to assess the drug susceptibilities of *Aspergillus fumigatus* isolates consisted of EUCAST and CLSI standardized broth microdilution techniques. Considering the increasing rate and the potential impact on the clinical outcome of azole resistance in *A. fumigatus*, more suitable techniques for routine testing are needed. The gradient concentration strip (GCS) method has been favorably evaluated for yeast testing. The aim of this study was to compare the CGS test with EUCAST broth microdilution for amphotericin B (AMB), posaconazole (PCZ), itraconazole (ITZ), voriconazole (VRZ), and isavuconazole (ISA). A total of 121 *Aspergillus* section *Fumigati* strains were collected, including 24 *A. fumigatus sensu stricto* strains that were resistant to at least one azole drug. MICs were determined using GCS and EUCAST methods. Essential agreement between the 2 methods was considered when MICs fell within ±1 dilution or ±2 dilutions of the 2-fold dilution scale. Categorical agreement was defined as the percentage of strains classified in the same category (susceptible, intermediate, or resistant) with both methods. Essential agreements with ±1 dilution and ±2 dilutions were 96.7, 93.4, 90.0, 89.3, and 95% and 100, 99.2, 100, 97.5, and 100% for AMB, PCZ, ITZ, VRZ, and ISA, respectively. Categorical agreements were 94.3, 86.1, 89.3, and 88.5% for AMB, PCZ, ITZ, and VRZ, respectively. Detection of resistance was missed with the GCS for one strain (4.1%) for PCZ and for 2 strains (8.3%) for ISA. Determination of ITZ MICs using the GCS allowed the detection of 91.7% of azole-resistant strains. The GCS test appears to be a valuable method for screening azole-resistant *A. fumigatus* clinical isolates.

KEYWORDS antifungal susceptibility testing, EUCAST, gradient concentration strip, *Aspergillus fumigatus*, CYP51A, azole resistance

Aspergillus spp. are responsible for a wide spectrum of diseases, including allergic manifestations and chronic and invasive infections (1). *Aspergillus fumigatus* is the predominant causative species whatever the clinical form. Antifungal therapy is required for invasive and chronic forms and, in a more debated manner, immunoallergic forms (2, 3). When antifungal therapy is required, first-line therapy relies on azole drugs. According to different published guidelines, voriconazole (VRZ) and isavuconazole (ISA) are recommended for invasive aspergillosis, while itraconazole (ITZ) can be used for chronic pulmonary aspergillosis and allergic bronchopulmonary aspergillosis (2, 4, 5). In

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addition, posaconazole (PCZ) has been proven to be efficient for the prevention of invasive aspergillosis in patients with hematological malignancy with the highest risk of developing invasive aspergillosis (6, 7). Liposomal amphotericin B (AMB) can also be used for invasive aspergillosis in cases of failure or intolerance of voriconazole. Basically, *A. fumigatus* exhibits natural susceptibility to both the azoles and polyenes. However, some cryptic species of *Aspergillus* section *Fumigati*, often misidentified in routine practice as *A. fumigatus*, have increased MICs to azole drugs (8). Even more concerning, acquired azole resistance in *A. fumigatus* is now reported worldwide, with the highest incidences in the Netherlands and the United Kingdom (9). In a recent report, it was shown that azole resistance negatively impacts the prognosis for infected patients, with a 21% increased mortality rate in patients with azole-resistant strains compared to those infected with azole-susceptible strains (10). This led an international committee to modify the therapeutic strategy in such cases (11). Therefore, rapid detection of resistance to these drugs is mandatory for therapeutic adaptation. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) have set up broth microdilution methods to determine the MICs of antifungal drugs against molds (12). Clinical breakpoints (CBPs) for *A. fumigatus* have been defined by the EUCAST for AMB, VRZ, ITZ, PCZ, and ISA (13, 14). These methods have high inter- and intralaboratory agreement, but they are labor-intensive and time-consuming and require experienced technicians. Thus, techniques more suitable for routine testing are needed. An MIC strip test based on the diffusion of a gradient of drug concentrations from a strip has been shown to be a valuable alternative to reference methods in different settings, notably for testing yeast species (15). However, until now, few studies have evaluated its usefulness for testing antifungal susceptibility of filamentous fungi (16, 17). To our knowledge, most studies focused on *A. fumigatus* and comparing the MIC gradient concentration strip (GCS) with broth microdilution techniques were conducted using the CLSI method as a reference, and only rare studies, involving few strains ($n = 15$, $n = 20$, and $n = 24$, respectively) of *A. fumigatus*, used the EUCAST method as a reference (18–20). In the present study, we compared the MICs of azole derivatives, namely, ITZ, VRZ, PCZ, and ISA, and of amphotericin B (AMB) against a large panel of *A. fumigatus* clinical isolates, exhibiting different profiles of antifungal susceptibility and resistance, using the EUCAST method and the gradient concentration strip test.

RESULTS AND DISCUSSION

Figure 1 shows the distribution of MICs for the 5 drugs tested according to the method used. Overall, the distributions were very similar, leading to essential agreements within ±1 dilution of 96.7, 93.4, 90.9, 89.3, and 95.0% and within ±2 dilutions of 100%, 99.2%, 100%, 97.5%, and 100% for AMB, POS, ITZ, VOR, and ISA, respectively (Table 1). Except for PSZ, these results support data from Buil et al., who tested 68 *A. fumigatus* strains and found essential agreements of 91%, 96%, 43%, and 74% for POS, ITZ, VOR, and ISA, respectively (21). A slightly wider distribution of AMB MICs was found using the GCS method, as was previously mentioned for yeasts (22), but none of our strains could be considered resistant. The GCS test tended to shift PCZ and ITZ MICs toward slightly higher values, while VCZ and ISA MICs appeared slightly lower than those determined with the EUCAST method. The overestimation of PCZ MICs was mentioned previously by Idelevich et al., while Buil et al. found an even more marked trend for VCZ and ISA, with strip test results consistently being 2 to 3 steps lower than EUCAST results (19, 21).

The EUCAST, but not the CLSI, provides clinical breakpoints for *A. fumigatus* against the drugs tested in our study, allowing the categorization of the strains as susceptible, intermediate, or resistant (23). With the exception of ISA, we used these values for both techniques to evaluate the categorical correlation between the 2 methods (Table 2). Indeed, as a single value (1 mg/liter) is given for the differentiation between ISA-susceptible and -resistant isolates, we did not analyze major discrepancies for this drug. For AMB, only minor errors (strains classified as intermediate by one method and

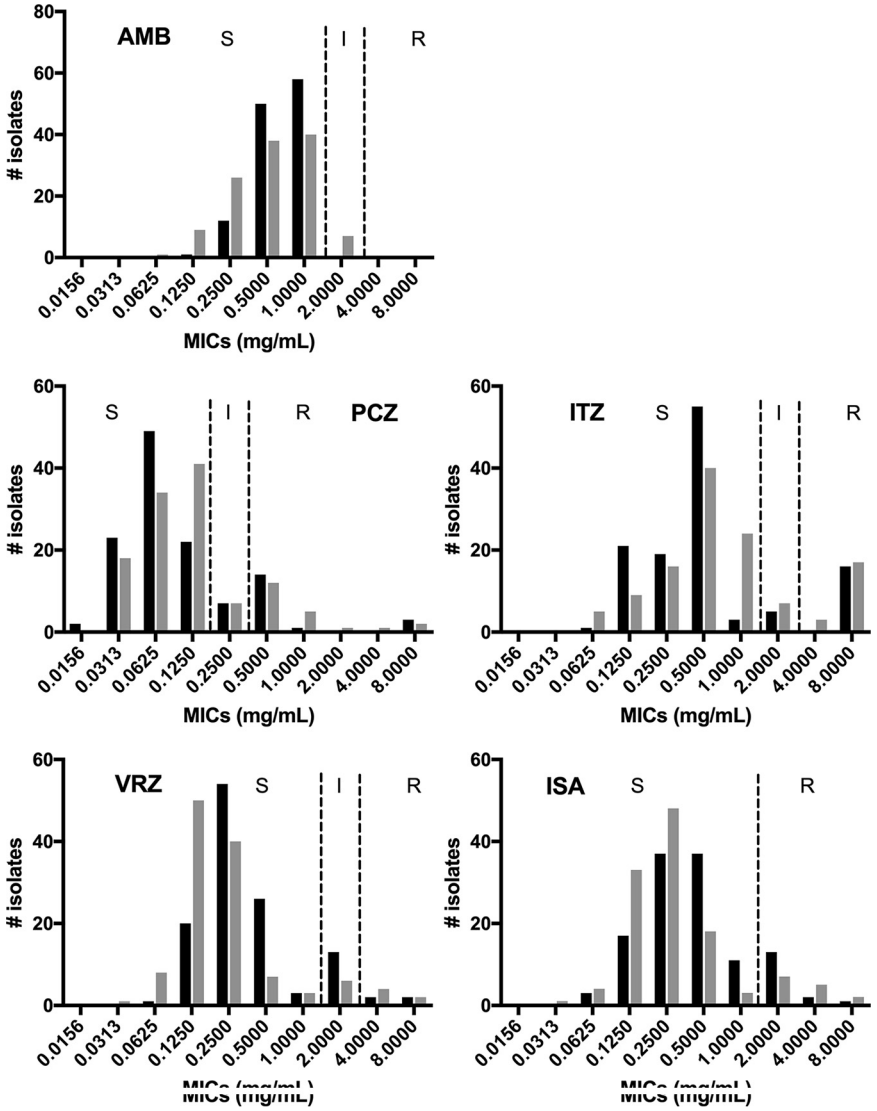

FIG 1 Distribution of MICs against amphotericin B and azole drugs according to the testing method (EUCAST [in black] and GCS [in gray]). Dotted lines delineate the following categories: susceptible (S), intermediate (I), and resistant (R).

susceptible by the other one) were observed, with a categorical agreement of 94.2%. Quite similar results were observed for ITZ and VRZ but nevertheless with the occurrence of one major error (strain determined to be resistant using the strip test but categorized as susceptible by the EUCAST method) for two separate strains. Categorical

TABLE 1 Distribution of strains according to the MIC values determined using a strip test compared to the MICs obtained with the EUCAST method[a]

Drug	No. of strains with MICs that fall within a $\log_2$ dilution difference of:							% essential agreement	
	≥−3	−2	−1	0	1	2	≥3	±2 dilutions	±1 dilution
AMB	0	3	32	65	20	1	0	100.0	96.7
PCZ	0	2	19	53	40	6	1	99.2	93.4
ITZ	0	4	9	60	41	7	0	100.0	90.9
VCZ	3	11	44	46	15	2	0	97.5	89.3
ISA	0	4	40	65	10	2	0	100	95.0

[a]Comparisons were done after the transformation of MIC values into $\log_2$ dilutions. AMB, amphotericin B; PCZ, posaconazole; ITZ, itraconazole; VRZ, voriconazole; ISA, isavuconazole.

TABLE 2 Categorical agreement according to MICs determined using the strip test and the EUCAST method[a]

Category	No. (%) of strains tested against:			
	AMB	PCZ	ITZ	VRZ
All strains ($n = 121$)				
Agreement	114 (94.2)	105 (86.8)	108 (89.3)	107 (88.4)
Discrepancies				
Minor	7 (5.8)	12 (9.9)	12 (9.9)	13 (10.7)
Major	0 (0)	3 (2.5)	1 (0.8)	1 (0.8)
Very major	0 (0)	1 (0.8)	0 (0)	0 (0)
Azole-resistant strains ($n = 24$)				
Agreement	ND	16 (66.7)	18 (75)	12 (50.0)
Discrepancies				
Minor	ND	5 (20.8)	5 (20.8)	11 (45.8)
Major	ND	2 (8.3)	1 (4.2)	1 (4.2)
Very major	ND	1 (4)	0 (0)	0 (0)

[a]See Materials and Methods for the definitions of categorical agreement. ND, not determined.

agreements for these drugs were calculated to be 89.3 and 88.4%, respectively. Finally, a slightly lower agreement, at 86.1%, was observed with PCZ, with 3 major errors and 1 very major error. The antifungal patterns observed with the EUCAST and GCS methods for the strains with major or very major discrepancies are presented in Table 3. These results are overall in the same range as those of Buil et al., who recorded major and very major errors for 2.9%, 2.3%, and 4.3% and for 6.0%, 6.5%, and 0% of strains for ITZ, PCZ, and VRZ, respectively (21).

Therefore, the setting of clinical breakpoints specifically for GCS tests, notably for PCZ, could be discussed. For example, shifting current CBPs for ITZ to a single point at 4 mg/ml would increase the categorical agreement in our study from 89.3% to 95.9%, supporting, by the way, the use of media containing 4 mg/ml ITZ for the routine screening of resistant isolates (21). However, shifting the CBP for PCZ to 0.5 mg/ml would not modify the categorical agreement, while it would remove very major errors but increase the number of major errors to 4. Setting clinical breakpoints specifically for GCS tests is also supported by existing epidemiological cutoff values (ECVs) specifically determined for the GCS and EUCAST methods (24, 25). In our study, rates of non-wild-type (WT) isolates identified with method-specific ECVs for the GCS versus the EUCAST method were 38.9 versus 0%, 17.4 versus 14.8%, 22.3 versus 13.2%, and 12.4 versus 14.0% for AMB, PCZ, ITZ, and VRZ, respectively. The most important discrepancies were observed with AMB ECVs, a phenomenon that may be related to substantial changes depending on the incubation time, 24 versus 48 h in our study (12).

Using the EUCAST breakpoints for both the GCS test and the EUCAST method, we found 24 strains to be resistant to at least one azole drug. Using these strains, essential agreement for the azole drugs remained high, at between 95.8 and 100% (data not shown). Similarly, Denardi et al. studied 15 ITZ-resistant *A. fumigatus* strains and found essential agreements between the GCS and EUCAST methods of 100%, 93%, and 87%

TABLE 3 Antifungal profiles of six *A. fumigatus* isolates presenting major and very major discrepancies in their categorization using the EUCAST and GCS methods[a]

CYP51A genotype	Antifungal profile			
	ITZ	VRZ	PCZ	ISA
WT	**S/R**	S/S	**S/R**	S/S
TR34/L98H	R/R	**S/R**	R/R	S/S
G54W	R/R	I/S	R/R	**R/S**
WT	S/S	S/S	**S/R**	S/S
TR34/L98H	R/R	R/I	**S/R**	R/R
F46Y,M172V,N248T,D255E,E427K	S/S	S/S	**R/S**	**R/S**

[a]Major and very major errors are in boldface type and in boldface and underlined type, respectively. S, susceptible; R, resistant; I, intermediate.

for VRZ, ITZ, and PCZ, respectively (18). However, categorical agreements were overall lower for all the azole drugs, ranging from 50 to 75% (Table 2). Looking at the *CYP51A* genotype, we found the wild-type genotype ($n = 4$) and TR34/L98H ($n = 14$), F46Y,M172V,N248T,D255E,E427K ($n = 3$), G54W ($n = 2$), and P216L ($n = 1$) mutations. All these mutations have been previously described (26). Three out of four wild-type strains were found to be resistant to ITZ with the GCS test but had a susceptible ($n = 2$) or intermediate ($n = 1$) profile using the EUCAST method (Table 3). This could suggest either undetected resistance with the EUCAST method or an overestimation of MICs based on the GCS method for ITZ. For the former, one can remember the existence of azole resistance without *CYP51A* mutations but rather associated with mutations in the *hapE* transcription factor (27). Nevertheless, an overestimation of the ITZ MICs with the GCS method compared to the CLSI method has already been reported (28). The fourth *CYP51A* wild-type strain was resistant to PCZ and VRZ only with the GCS test and was intermediate and resistant to ITZ with the EUCAST and GCS methods, respectively.

In both our study and the literature, the TR34/L98H mutation is the most commonly detected mutation in the case of azole resistance in *A. fumigatus*. It has been demonstrated in large isolate collections that TR34/L98H isolates were always resistant to ITZ according to the EUCAST CBPs (29, 30). In our study, all but two resistant strains were resistant to at least ITZ (91.7%). The two ITZ-susceptible strains had a less common CYP51A genotype (F46Y,M172V,N248T,D255E,E427K). The categorical agreement for classifying TR34/L98H isolates as resistant was 100% for ITZ and ISA but showed discrepancies with PCZ (1 minor error and 1 major error) and VRZ (8 minor errors and 1 major error). In addition, the distribution of ITZ MICs clearly distinguishes between susceptible and resistant isolates, in contrast to the other azole drugs, for which the distribution of MICs is more interspersed. A previous study reported a TR34/L98H strain showing intermediate susceptibility to ITZ with the GCS (MIC = 1.5 mg/ml), while the strain was considered resistant with the EUCAST method (MIC > 8 mg/liter) (23). The number of resistant strains included in our study is quite limited, and additional studies are required to confirm that the determination of ITZ MICs, including using the GCS method, is reliable for the characterization of the azole resistance phenotype.

In conclusion, considering the spread and clinical consequences of azole resistance in *A. fumigatus*, a rapid and reproducible technique to screen for resistance is needed. The significant cost and the current lack of specific CBPs are the main drawbacks of the GCS method, warranting further studies aimed at testing other alternative techniques, such as the disk diffusion method (31). However, our results support the use of the GCS method as an alternative to the EUCAST reference method. Additional studies on the need for specific CBPs adapted to the GCS method are warranted.

MATERIALS AND METHODS

Strains. We performed a retrospective multicentric study from 4 university hospitals in Paris, France. A panel of 121 *Aspergillus* section *Fumigati* clinical strains was selected, whose identification had been done previously based on macroscopic and microscopic morphological examinations. Among these strains, 24 were previously found to have reduced susceptibility to one of the antifungal drugs tested in this study using the GCS method. These strains had their identification as *A. fumigatus sensu stricto* confirmed after direct sequencing of a fragment encoding β-tubulin (8). All strains were stored at −80°C until testing.

Antifungal susceptibility testing. We used the EUCAST broth microdilution method for conidium-forming molds, version EDef 9.3, to determine the MICs of antifungal agents (13, 23). The MIC was considered the lowest concentration that completely inhibited the growth of the strains. Reading was done visually at 48 h, and the MIC was considered the lowest concentration that completely inhibited growth. *Candida krusei* strain ATCC 6258 was used as a quality control in each series. For the strip tests, Etest strips (bioMérieux, Craponne, France) were used for AMB, PCZ, VRZ, and ITZ, and strips loaded with ISA were purchased from Liofilchem (Roseto degli Abruzzi, Italy). For both azole drugs and AMB, concentrations of antifungal drugs on the strips ranged from 0.002 to 32 mg/liter. Plates of RPMI 1640 agar supplemented with morpholinepropanesulfonic acid (MOPS) and glucose (bioMérieux) were inoculated with conidial solutions at a 0.5 McFarland standard in saline with Tween 80 with a sterile cotton swab. Plates were then incubated at 35°C and read at 24 and 48 h. MIC values were defined as the lowest concentration of the antifungal drug at which the elliptical zone of growth inhibition intersected the strip (100% inhibition). For both methods, the *A. fumigatus* reference strain ATCC 204305 was used for quality control.

Interpretation of results. To directly compare the MICs obtained with the 2 methods, the MICs obtained with the GCS were adjusted to the nearest highest 2-fold dilution value that matched the EUCAST 2-fold dilution scheme. Essential agreement between the results of both methods was considered when the MIC values obtained fell within ±1 dilution and ±2 dilutions of the 2-fold dilution scheme. Clinical breakpoints determined by the EUCAST were used for both the strip test and EUCAST results to categorize the strains as susceptible, intermediate, and resistant (13, 23). For AMB, ITZ, and VRZ, susceptibility and resistance are defined by MICs of ≤1 mg/ml and >2 mg/ml, respectively, and for PCZ, the values are 0.125 and 0.25 mg/ml, respectively, while a single value of 1 mg/ml differentiates between strains that are susceptible and resistant to ISA. Categorical agreement was defined as the percentage of strains classified in the same category with both methods. Discrepancies were classified as (i) very major if an isolate classified as resistant by the reference method was categorized as susceptible by the strip test, (ii) major if an isolate classified as susceptible by the reference method was classified as resistant by the strip test, and (iii) minor when susceptible versus intermediate, resistant versus intermediate, intermediate versus susceptible, or intermediate versus resistant discrepancies were observed, as previously described (15). As a single CBP value is given for ISA, only minor discrepancies could be identified for this drug (23). Method-specific epidemiological cutoff values (ECVs) were previously defined to distinguish between wild-type (WT) and non-WT isolates using MIC values of ≤1 mg/ml, ≤0.25 mg/ml, ≤1 mg/ml, and ≤1 mg/ml for AMB, PCZ, ITZ, and VRZ, respectively, using the EUCAST method (14, 32, 33) and using MIC values of ≤0.5 mg/ml, ≤0.25 mg/ml, ≤2 mg/ml, and ≤0.5 mg/ml, respectively, when testing the drugs using the GCS method (24, 25). We used these values to analyze the rate of non-WT isolates in our panel based on (i) EUCAST MICs interpreted by using EUCAST-specific ECVs and (ii) GCS MICs interpreted by using GCS-specific ECVs.

CYP51A gene sequencing. Strains with an MIC suggestive of resistance, whether this was determined with the GCS test or the EUCAST method, had their CYP51A gene sequenced. Briefly, strains were cultured overnight at 35°C in Sabouraud broth with shaking (1,500 rpm). DNA was extracted using thermal lysis followed by Chelex-based purification (34). Amplification and direct sequencing were performed using primers previously described by Mellado et al. (35). After editing, the sequences were compared to the sequence of the wild-type reference strain Af293 (GenBank accession number XM_747044).

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