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Time of Incubation for Antifungal Susceptibility Testing of *Aspergillus fumigatus*: Can MIC Values Be Obtained at 24 Hours?▽

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A collection of *Aspergillus fumigatus* isolates was used to check if MICs can be read at 24 h. At 24 h, the geometric mean MIC of itraconazole for resistant isolates was determined to be 5.11 mg/liter, but the MIC was read as 16 mg/liter at 48 h. At 24 h, MICs for 51.5% of resistant strains were determined to be ≤2 mg/liter. MICs must be obtained at 48 h.

The incidence of invasive mold infections has increased in the last decades. The mortality due to these infections is very high, and outcomes are determined mainly by the risk factors of the patients. Although the correlation between the results of antifungal susceptibility testing and the outcomes for patients is far from perfect, several reports have described poorer responses to therapy by those infections caused by isolates for which MICs are high. Lass-Flörl et al. (11) reported favorable outcomes for 20% of patients with invasive disease caused by *Aspergillus terreus* who were treated with amphotericin B, in comparison with 47% of amphotericin B-treated patients with disease caused by other *Aspergillus* spp. The amphotericin B MICs for *A. terreus* have been shown to be higher than those for *A. fumigatus* (11, 20), and therefore, the poorer responses of patients were attributed to higher amphotericin B MICs. In addition, resistant strains of *A. fumigatus* have recently been reported (2–5, 8, 13, 14, 21).

Antifungal susceptibility testing has been standardized for molds (16). The optimal susceptibility testing conditions for the detection of azole-resistant strains were defined by Espinel-Ingroff et al. (6). The recommendation was to estimate the end point as the complete inhibition of visual fungal growth at 48 h. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) started the standardization of the antifungal susceptibility testing of molds, and now the standard is pending final approval (10). In previous work (7), we observed that the time of incubation significantly affected the final MIC readings and that sometimes a major increase (two to six dilutions) occurred between readings at 24 and 48 h. Most *A. fumigatus* strains are capable of growth in RPMI 1640 within 24 h. Thus, there is a chance to obtain end points at 24 h and deliver the results to the clinicians at an earlier time.

The aim of this study was to check if the MICs of antifungal drugs for *A. fumigatus* can be read at 24 h instead of at 48 h. To achieve this, we have compared a large collection of clinical isolates of *A. fumigatus* for which MICs of azole drugs vary from low to high.

Microorganisms. One hundred fifty itraconazole-susceptible *A. fumigatus* strains and 33 *A. fumigatus* strains resistant to itraconazole were included. The mechanisms of resistance of these strains have been described previously (5, 12–15).

Antifungal susceptibility testing. Microdilution testing was performed by following the EUCAST methodology (10). This standard is similar to approved standard M38-A published by the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) (16), with the following modifications: (i) RPMI 1640 is supplemented with glucose to reach a 2% concentration, (ii) the inoculum size is between 2×10^5 and 5×10^5 CFU/ml, and (iii) inocula are prepared by counting spores in a hematocytometer (1, 18, 19). *A. fumigatus* ATCC 204305 and *A. flavus* ATCC 204304 were used as quality control strains (16).

The antifungal agents used were amphotericin B (range, 0.03 to 16 mg/liter; Sigma Aldrich Química), itraconazole (range, 0.015 to 8 mg/liter; Janssen S.A.), voriconazole (range, 0.015 to 8 mg/liter; Pfizer S.A.), ravuconazole (range, 0.015 to 8 mg/liter; Bristol-Myers Squibb), and posaconazole (range, 0.015 to 8 mg/liter; Schering-Plough Research Institute).

End points were recorded at 24 and 48 h and defined as the antifungal concentration that produced a complete inhibition of visual growth. MICs for resistant strains were determined at least two times on different days (range of repetitions, 2 to 11).

Statistical calculations. Statistical analysis was done with the Statistical Package for the Social Sciences (version 15.0; SPSS S.L). Both on-scale and off-scale results were included in the analysis. The off-scale MICs were converted to the next concentration up or down.

Table 1 shows the geometric mean (GM) MICs and the ranges obtained at 24 and 48 h for susceptible and itraconazole-resistant isolates. For all itraconazole-resistant isolates, the GM MIC of itraconazole was 16 mg/liter at 48 h. However, at 24 h the GM MIC was 5.11 mg/liter, and itraconazole MICs for some strains were as low as 0.25 mg/liter. This result was not isolate dependent but rather due to the poor reproducibility of MICs read at 24 h for itraconazole-resistant strains. As

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TABLE 1. GM MICs and ranges of MICs (mg/liter) obtained at 24 and 48 h for azole drug-susceptible and -resistant *A. fumigatus* isolates

Antifungal	Reading time (h)	GM MIC (range) for:	
		Susceptible isolates (n = 150)	Resistant isolates (n = 33)
Amphotericin B	24	0.14 (0.03–1)	0.14 (0.06–0.5)
	48	0.30 (0.12–2)	0.29 (0.06–1)
Itraconazole	24	0.16 (0.06–0.5)	5.11 (0.25–16)
	48	0.33 (0.12–1)	16 (16–16)
Voriconazole	24	0.29 (0.03–1)	0.81 (0.12–8)
	48	0.56 (0.06–2)	1.6 (0.12–8)
Ravuconazole	24	0.32 (0.06–1)	1.13 (0.12–8)
	48	0.60 (0.12–2)	2.4 (0.12–16)
Posaconazole	24	0.04 (0.015–0.12)	0.41 (0.06–16)
	48	0.08 (0.03–0.5)	0.83 (0.25–16)

the testing of all isolates was repeated at least two times, the analyses of the MICs obtained at 24 h showed that MICs for 17 (51.5%) of 33 itraconazole-resistant strains were read as ≤2 mg/liter at least once. For susceptible strains, the 24-h GM was 0.16 mg/liter and the 48-h GM was 0.33 mg/liter (Table 1). For voriconazole, ravuconazole, and posaconazole, the differences were less striking, and GMs showed one twofold-dilution difference between 24- and 48-h readings (Table 1) for all strains tested. As exceptions, there was one isolate not growing at 24 h and MICs of posaconazole for two further isolates were shown to be 2 mg/liter at 24 h and 16 mg/liter at 48 h.

In this study, the MICs for a large collection of susceptible and itraconazole-resistant strains of *A. fumigatus* have been analyzed and the results obtained at 24 and 48 h have been compared. Several articles have associated poor outcomes for patients with infections caused by isolates or species for which MICs are high (9, 11, 17, 20). Therefore, the quick detection of resistant isolates can influence the outcome for the patient, allowing a prompt change of the antifungal treatment. The growth of most clinical isolates of *A. fumigatus* is apparently adequate at 24 h in antifungal susceptibility testing microplates with RPMI 1640–2% glucose. Furthermore, we have used an inoculum size of 2×10^5 to 5×10^5 CFU/ml, 10-fold larger than the one recommended by CLSI document M38-A. This inoculum provided better growth of *A. fumigatus* than smaller inocula, making the visual estimation of the end point straightforward. Our results do no support reading end points at 24 h because MICs for 51.5% of the itraconazole-resistant strains were determined to be ≤2 mg/liter at least once at that time point. As itraconazole MICs for some isolates were as high as 16 mg/liter at 24 h, one may consider the possibility of accepting the results for these isolates at 24 h. However, we detected such high MICs of itraconazole for two isolates at 24 h but posaconazole MICs of 2 mg/liter at 24 h and 16 mg/liter at 48 h.

In summary, the detection of resistant strains of *A. fumigatus* by means of EUCAST-standardized antifungal susceptibility testing for molds requires 48 h of incubation. Too many false-negative results for resistant strains were detected at 24 h, making MIC reading at this time point unreliable.

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