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Antifungal susceptibility testing in *Aspergillus* spp. according to EUCAST methodology

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The availability of new antifungal agents has multiplied the demand for *in vitro* antifungal susceptibility testing for *Aspergillus* spp. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has charged its Antifungal Susceptibility Testing Subcommittee (AFST-EUCAST) with the preparation of new guidelines for *in vitro* susceptibility testing of antifungals against *Aspergillus* spp (EUCAST-AST-ASPERGILLUS). This committee has modified the reference method for broth dilution antifungal susceptibility testing of filamentous fungi (M38-A) as follows: (i) RPMI 1640 supplemented with 2% glucose (RPMI 2%G) as assay medium, (ii) inoculum preparation by conidium counting in a haemocytometer and (iii) an inoculum size of 2×10^5–5×10^5 CFU/ml. The incubation time is about 48 h at 35°C and MIC is read visually. The MIC value is a no-growth visual endpoint. The standard method described herein is intended to provide a valid and economic method for testing the susceptibility to antifungal agents of *Aspergillus* spp., to identify resistance, and to facilitate an acceptable degree of conformity, e.g. agreement within specified ranges and between laboratories in measuring the susceptibility.

Keywords susceptibility tests, EUCAST, resistance, *Aspergillus*

Introduction

Invasive aspergillosis has emerged worldwide as an important cause of infection among patients undergoing cancer chemotherapy, hematopoetic stem-cell transplantation, or solid organ transplantation [1–3]. The crude mortality from invasive aspergillosis is around 85% and falls to around 50% if treated [3–5]. Until 1990 there was only one drug useful for treatment of *Aspergillus* disease, amphotericin B, which has to be given intravenously and has a number of serious toxicities. In the meantime, several other drugs are available for treatment options for invasive aspergillosis such as voriconazole, posaconazole and caspofungin [4–6].

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Aspergillus fumigatus is most frequently isolated from clinical specimens, but other important species include *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus terreus* [1].

Antifungal susceptibility testing remains less well developed and utilized than antibacterial testing, the scientific support for its validity has benefited greatly by extrapolation from antibacterial testing. Knowledge of mechanisms of antifungal resistance has been valuable in identifying resistant isolates and using them to validate *in vitro* measurements systems [7]. The vast majority of isolates seems to be susceptible to the common drugs [8–11], yet MIC (minimum inhibitory concentration) data lack of *in vivo* correlation. There are many different methods of determining *in vitro* susceptibilities [12–14]; currently, the M38-A reference method for filamentous fungi, published by the Clinical Laboratory Standard Institute (CLSI) is available for the determination of MICs of *Aspergillus* spp. against antifungals [15]. This methodology exhibits

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some limitations and it is well known that the size of inoculum [16], the type of growth medium [17], the time of incubation and the inoculum preparation method can influence MIC values [11,18]. Several studies showed the importance of inoculum preparation in haemocytometer for accurate and reproducible preparation independent of the colour and size of conidia [11,19,20].

Using a collection of *A. fumigatus*, Denning and coworkers have made substantial progress in this area [13]. Two isolates were collected from patients who did not respond to therapy with itraconazole. These isolates were resistant to itraconazole in a murine model of invasive aspergillosis and had elevated itraconazole MICs. The choice of assay system (10^6 conidia as inoculum, 2% 1640 RPMI supplemented with glucose) was critical in the detection of these elevated MICs. Inclusion of glucose in the RPMI broth enhanced growth and facilitated the determination of endpoints. Based on these findings the European Committee on Antimicrobial Susceptibility Testing (EUCAST) has charged the Subcommittee on Antifungal Susceptibility Testing (AFST-EUCAST) with preparation of guidelines for *in vitro* susceptibility testing of antifungals against *Aspergillus* spp. This committee adopted a modified M38-A reference method and developed a proposed EUCAST broth dilution method for susceptibility testing against *Aspergillus* (EUCAST-AST-ASPERGILLUS).

Herein we will give an overview on the issues that are important for testing in the clinical laboratory with special emphasis on the draft of the EUCAST-AST-ASPERGILLUS methodology.

Methods for susceptibility testing for *Aspergillus*

CLSI M38-A Reference Method

Direct adaptations of the M27-A methodology to *Aspergillus* were shown to generate reproducible results [10,21]. Following the principles established for testing yeasts, a standard method entitled 'Reference method for broth dilution antifungal susceptibility testing of filamentous fungi' has been published as CLSI M38-A [15]. Test conditions are given in detail in Table 1. This technique is characterized by a high reliability and reproducibility and has become a tool for researchers. However, the M38-A methodology exhibits some limitations as mentioned above and the clinical usefulness is somewhat controversial [12,22,23]. The standard method lacks any discrimination of amphotericin B-resistant isolates from susceptible ones. Modifications in the test medium have been proposed to solve this problem. However, there is yet no consensus about this issue. While the use of antibiotic medium 3 as the test medium instead of RPMI 1640 has highlighted the resistant isolates in the hands of some investigators; the results obtained by others have been contradictory [21,22].

Table 1 CLSI and EUCAST conditions for antifungal susceptibility testing

Characteristic	CLSI M38A	EUCAST-*Aspergillus*
Suitability	Conidium and spore forming fungi	*Aspergillus* species
Inoculum	0.4×10^4–5×10^4 CFU/ml	2–5×10^5 CFU/ml
Inoculum standardization	Spectrophotometrically	Haemocytometer
Test medium	RPMI 1640	RPMI 2% glucose
Format	Microdilution	Microdilution
Temperature	35°C	35°C
Duration of incubation	48 h	48 h
Endpoint	No growth	No growth

Recently, the CLSI Subcommittee for Antifungal Susceptibility Tests conducted two collaborative studies and identified conditions for the determination of minimal fungicidal concentration (MFC) endpoints for mould isolates, but the clinical relevance of these *in vitro* endpoints in patient management needs to be investigated [24,25]. The poor *in vitro* fungicidal activity of amphotericin B appears to correspond with the refractory nature of *A. terreus* infections [26,27]. However, all of the issues of standardization that occur with MICs also apply to MFCs. Many variables such as size of inoculum, incubation period, drug carry over, sample volume, and end point influence the test outcome [28].

EUCAST-AST-ASPERGILLUS draft 1

The standard method described herein is intended to provide a valid, easy, rapid and economic method for testing the susceptibility to antifungal agents of *Aspergillus* spp., to identify resistance, and to facilitate an acceptable degree of conformity, e.g. agreement within specified ranges and between laboratories in measuring the susceptibility. The use of (i) RPMI 1640 supplemented with 2% glucose (RPMI 2%G) as assay medium, (ii) the inoculum preparation by conidium counting in a haemocytometer and (iii) an inoculum size of 2×10^5–5×10^5 CFU/ml display the important differences between CLSI and EUCAST, see Table 1.

In general, the use of stock solutions, antifungal agents, range of concentrations tested, and the quality

control procedures are in accordance with M38-A protocol [15]. The method for preparing the plates with hydrophilic and hydrophobic antifungal agents is described in detail in the EUCAST Discussion E.Dis.7.1 for fermentative yeasts [29]. Amphotericin B, itraconazole, voriconazole, posaconazole and flucytosine are recommended for *in vitro* susceptibility testing.

Assay medium and microtitration plates

RPMI 1640 (with glutamine and a pH indicator but without bicarbonate) supplemented with 2% glucose (RPMI 2% G) and 3-(N-morpholino) propanesulfonic acid (MOPS) at a final concentration of 0.165 mol/l, pH 7.0 is recommended. The medium used to prepare the plates is double strength to allow for a 50% dilution once the inoculum is added.

Preparation of inoculum

Inoculum suspensions are prepared from fresh, mature (2- to 5-day-old) cultures grown on potato dextrose agar slants at 35°C. In some cases an extended incubation is required for proper sporulation of the isolate. Colonies are covered with approximately 1 ml of sterile water supplemented with 0.1% Tween 20. The conidia are collected carefully with a sterile cotton swab and transfered to a sterile tube; homogenize this suspension for 15 seconds with a gyratory vortex mixer at 2,000 rpm and perform appropriate dilutions with sterile water for counting in a haemocytometer and check for hyphae and clumps. Filter the inoculum through a sterile nylon net filter with pore sizes of 11 μm in the presence of a significant number of hyphae or clumps ($>5\%$ of fungal structures). This step may be repeated as many times as necessary. The suspension is then adjusted with sterile distilled water to $2–5 \times 10^6$ CFU/ml by counting the conidia in a haemocytometer. This suspension is then diluted further 1:10 with sterile distilled water to obtain a final working inoculum of $2–5 \times 10^5$ CFU/ml. All adjusted suspensions should be quantified by plating on Sabouraud dextrose agar plates.

Inoculation of microtitration plates

Each well of a microtitration tray is then inoculated with 100 μl of $2–5 \times 10^5$ CFU/ml conidial suspension, which brings the drug concentration and inoculum density to the final desired concentrations (final inoculum $= 1 \times 10^5–2.5 \times 10^5$ CFU/ml). The growth control wells contain 100 μl of sterile drug-free medium and inoculated with 100 μl of the same inoculum suspension. The microtitration plate is incubated static, in a humid atmosphere in a sealed container or bag at 35°C for 48 h.

Reading results and interpretation of results

The endpoint is read visually, recording the level of growth for each well, using a viewing mirror for visualization of fungal growth. The concentration of drug in the first well in which there is no growth is the MIC value. Single colonies at the surface should be ignored as should skip-wells (additional growth occurs above the determined MIC). Although interpretation of mould MICs has long been known to be problematic and interpretative breakpoints have not been established, isolates with itraconazole MICs of ≥ 8 μg/ml are certainly resistant, MICs of 0.125–0.5 μg/ml are clearly susceptible. These interpretations relate to correlative *in vivo* work, and genotypic determinations of resistance. Other itraconazole values and other drug MICs (including amphotericin B) require more validation before breakpoints can be recommended. No interpretative breakpoints for MFCs have been proposed.

Standard conditions are not described in the EUCAST document for the echinocandins. Several testing conditions influence the *in vitro* data for *Aspergillus* spp. and it is not known which medium is best suitable for susceptibility testing [10]. The term 'trailing' describes the reduced and persistent growth or turbidity which some isolates of *Aspergillus* above the echinocandin MIC [21]; trailing precludes an easy and reproducible MIC determination. This problem is alleviated by visually assessing, instead of the growth inhibition, the formation of compact, small microcolonies or 'clumps' in the bottom of the microdilution MIC well or the significant microscopic morphologic hyphal alterations (short, highly branched filaments with swollen germ tubes and distended, balloon-like cells). These morphologic changes were reported as minimum effective concentrations to distinguish them from MICs, yet, clinical resistance caused by isolates with decreased susceptibility to caspofungin has not been documented yet [21].

Gomez-Lopez *et al*. [18] did some studies according the EUCAST-AST-ASPERGILLUS draft. This method differentiated amphotericin B or itraconazole-resistant *Aspergillus* strains *in vivo* from the susceptible ones. The MICs of amphotericin B and itraconazole were >2 and >8 μg/ml, respectively. The interlaboratory reproducibility of the EUCAST draft is currently being evaluated. Recently, a multicenter study investigated 6 *Aspergillus* and *Candida* strains and prelimin-

Table 2 Evaluation of the EUCAST-AST-ASPERGILLUS. The table summarizes the interlaboratory reproducibility of the results for each drug/strain combination by an intraclass correlation coefficient (ICC) which compared the results of three repetitions in duplicate of the MICs obtained by each participant ($n=6$).

Strain	ICC
A. fumigatus 1	0.85
A. fumigatus 2	0.99
A. terreus 3	0.86
A. flavus 4	0.85
A. flavus ATCC 22019	0.91
A. fumigatus ATCC 204304	0.91

The correlation was evaluated by using the ICC, which was expressed to a maximum value of 1 and with a confidence interval of 95% (95% CI). The ICC is a reverse measurement of the variability, agreement represents concordance between values. This scale analysis exhibits the highest statistical power for correlation studies.

ary data show that the EUCAST method described herein is highly reproducible; excellent agreement was found for intralaboratory and interlaboratory reproducibility, see Tables 2 and 3.

Other antifungal susceptibility testing methods for moulds

Etest (AB Biodisk, Sweden) is a commercially available method for antimicrobial susceptibility testing. Etest is based on a combination of the concepts of dilution and diffusion tests. Like dilution methods, Etest directly quantifies antifungal susceptibility in terms of discrete MIC values. For *Aspergillus* spp., good correlations with amphotericin B and itraconazole Etest and M38-A method have been demonstrated [12,30,31]. Meletiadis *et al*. compared the results obtained by the Etest and the Sensititre Colorimetric Methods with the CLSI document M38-A [32]. By contrast, low levels of agreement between the CLSI and the Etest were found for most species, especially after 48 h of incubation. The choice of growth medium appears critical with the Etest technique, RPMI-based agars seem to be the most useful [30].

Table 3 Interlaboratory evaluation of the EUCAST-AST-ASPERGILLUS. The table summarizes the ranges and percentages of MIC values (μg/ml) of *A. flavus* ATCC 2043404 and *A. fumigatus* ATCC 204305. Three repetitions in duplicate were performed.

Drug	MIC range	MICs in the range (%)
A. fumigatus ATCC 204305		
AMB	0.25–1.0	100
ITR	0.12–0.50	100
VOR	0.25–1.0	94.4
POS	0.03–0.25	90.3
A. flavus ATCC 204304		
AMB	0.50–2.0	97.2
ITR	0.12–0.50	100
VOR	0.50–2.0	91.7
POS	0.12–0.50	91.7

AMB, amphotericin B; ITR, itraconazole; VOR, voriconazole; POS, posaconazole.

The use of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] or XTT (2,3-bis (2methoxy-4-nitro-5-dulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) as a colorimetric marker for redox potential has been found convenient for *Aspergillus* [33,34]. This approach generates MICs comparable to those in CLSI method and presents substantial opportunities for automation. Recently, the YeastOne Colorimetric Antifungal plate has been favourably compared to CLSI methodology with amphotericin B and itraconazole [35]. However, the reliability and clinical relevance should be further addressed.

Flow cytometry has also been known as a possible tool for antifungal susceptibility testing and has been developed for several studies [36]. Staining or lack of staining with suitable dyes permits the rapid detection of damaged fungi: this method can distinguish *Aspergillus* isolates susceptible to amphotericin B from those resistant [37]. In conceptually related studies, fluorescent viability dyes have been used to examine the nature of drug-induced damage and to estimate minimal fungicidal concentrations for Aspergilli [38].

All of these alternative methods in general correlate more or less with the standard method. However, each also has its own disadvantages. While colorimetric microdilution method is as cumbersome as the standard method, Etest is relatively expensive. Disk diffusion is the most attractive alternative method so far investigated [13]. It is not only easy to perform and cheap, but also well suited for routine use in mycology laboratories.

Frequency of *in vitro* resistance

The widespread use of antifungal therapy could lead to development of resistance as reports of itraconazole-resistant *A. fumigatus* isolates, cross-resistance and multidrug resistance have increased [14,39,40]. Although development of resistance during treatment to amphotericin B is rare, an increase in the incidence of infections caused by multiresistant species or species resistant to amphotericin B is evident [41–43]. Primary *in vitro* resistance to amphotericin B has been observed for *A. terreus* and *A. flavus*. Also, *Aspergillus* isolates recovered from patients who previously received amphotericin B exhibited higher MICs compared with isolates from patients without amphotericin B exposure [42]. The study of mechanisms of resistance to ampho-

Table 4 *In vitro* susceptibility of *Aspergillus* spp.: acquired and intrinsic resistance to itraconazole

Number of total isolates	Species	Number of resistant isolates (patients)	ITR resistance		Reference
			Acquired	Intrinsic	
107	*A. fumigatus*	4 (3)	4	0	[50]
156	*A. fumigatus*	4 (3)	2	2	[47]
17	*A. nidulans*	1 (1)	0	1	[47]
150	*A. fumigatus*	0	0	0	[51]
7	*A. fumigatus*	3 (2)	1	2	[14]

ITR, itraconazole.

tericin B has been hampered by contradictory reports regarding the correlation between *in vivo* outcome and *in vitro* data for *A. fumigatus* in experimental infections or in clinical cases and the difficulty in producing adequate laboratory mutants [27,44,45].

A number of itraconazole-resistant clinical isolates as well as spontaneous and induced mutants have been documented in *A. fumigatus*, *A. flavus,* and *A. nidulans,* with a maximum resistance rate of about 4.2% [14,46–48]. By contrast, the rate of *A. niger* azole resistance in the clinical culture collection was about 31% [49]. The reason for this high rate is unknown yet. Intrinsic and acquired itraconazole resistance is reported for *A. fumigatus* and *A. nidulans*, as shown in Table 4 [14,47,50,51]. Resistance has been associated with reduced accumulation of drug in *A. fumigatus* clinical isolate [13]; a point mutation in the gene *CYP51A* contributed to itraconazole resistance in clinical isolates and overexpression of the azole target has been postulated; these mechanisms have correlated with elevated MICs as compared with results in susceptible isolates with low MICs [52,53].

Resistance to posaconazole has also been attributed to point mutations in the *CYP51A* gene that encodes its target protein laboratory-selected mutants (*A. fumigatus*) with posaconazole reduced activity [54]. A degree of cross resistance appears to be between itraconazole and posaconazole as shown in a neutropenic murine model of invasive aspergillosis [55]. In an isolate with elevated voriconazole MICs, a single nucleotide change was detected in *CYP51Ap*, but not in isolates with low MICs.

Conclusion

Antifungal susceptibility testing has evolved rapidly during the last decade and has now become a relevant tool. To date, studies with moulds have concentrated on the selection of optimal *in vitro* conditions and little attention has been paid to correlation with clinical outcome. In order to be clinically useful, *in vitro* methods should predict *in vivo* response to therapy.

Currently, susceptibility testing is not an infallible answer to questions about treatment of *Aspergillus* infections and we are unable to speculate on breakpoints for clinical reporting, to establish these will require a large number of clinical isolates with varying degrees of resistance and known clinical outcome. After the EUCAST-AST- ASPERGILLUS protocol is finalized, breakpoints will be defined for *Aspergillus* following the procedure accorded by EUCAST that can be consulted at http://EUCAST.org.

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