

Comparison of the EUCAST-AFST broth dilution method with the CLSI reference broth dilution method (M38-A) for susceptibility testing of posaconazole and voriconazole against *Aspergillus* spp.

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ABSTRACT

The susceptibilities of 40 clinical isolates of *Aspergillus* spp. (*Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus terreus*) were determined for posaconazole and voriconazole by the CLSI M38-A and EUCAST-AFST broth dilution methods. Where a discrepancy was observed between the methods, the EUCAST method tended to give higher MIC values. Overall, the level of agreement was 92.5% and the intra-class correlation coefficient was >0.90.

Keywords *Aspergillus* spp., broth dilutions, posaconazole, susceptibility testing, voriconazole

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INTRODUCTION

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) is a standing committee organised by the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) and the national breakpoint committees of Europe. EUCAST was set up to harmonise breakpoints of antimicrobial agents and to standardise susceptibility testing in Europe so that comparable results and interpretations, both in clinical practice and in antimicrobial resistance surveillance, can be achieved. EUCAST has tasked the Subcommittee on Antifungal Susceptibility Testing (EUCAST-AFST) with preparing guidelines for testing antifungal agents against various fungal species.

EUCAST-AFST has already developed a standard broth microdilution procedure for the determination of antifungal MICs for fermentative species of yeasts (document E.Dis.7.1) [1]. This standard is based on the reference procedure described in document M27-A2 by the CLSI, formerly the NCCLS [2]. In addition, EUCAST-

AFST has developed a method for determining broth dilution MICs for *Aspergillus* spp. This is a microdilution method that is intended to produce concordant results with the CLSI reference method for antifungal susceptibility testing of filamentous fungi (document M38-A) [3]. The EUCAST method includes some modifications based on previous studies: (1) RPMI-1640 medium supplemented with glucose 2% w/v (RPMI 2% G) is used as the assay medium; (2) the inoculum is prepared by counting conidia in a haemocytometer; and (3) an inoculum size of 1×10^5 to 5×10^5 conidia/mL is used [4–6].

The agreement and correlation between the various methods has not been evaluated previously for any antifungal agent. The aim of the present study was to investigate the concordance obtained between the CLSI and EUCAST standard methods when determining the MICs of voriconazole and posaconazole for clinical isolates of *Aspergillus* spp.

MATERIALS AND METHODS

Test isolates

A panel of 40 clinical isolates belonging to four different *Aspergillus* spp. (ten each of *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus terreus*) was used. The isolates were stored at -70°C and were subcultured twice on

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potato dextrose agar slants at 35°C for 2–3 days before testing. Two reference strains, *Candida krusei* ATCC 6258 and *Candida parapsilosis* ATCC 22019, were included as quality controls, with MICs being within the accepted limits by both methods on all occasions [7].

Antifungal agents

Posaconazole (Schering-Plough, Kenilworth, NJ, USA) and voriconazole (Pfizer, New York, NY, USA) were provided in the form of powders. Both drugs were dissolved in 100% dimethylsulphoxide to make stock solutions of 3.2 g/L, which were stored at –70°C until required. The range of posaconazole and voriconazole concentrations tested was from 0.015 to 8 mg/L.

EUCAST broth dilution method

The assay medium used was RPMI-1640 (Sigma-Aldrich, Stockholm, Sweden), with glutamine but without bicarbonate, buffered to pH 7.0 with 0.165 M morpholinopropanesulphonic acid (Sigma-Aldrich) and supplemented with glucose 2% w/v. Culture medium was prepared as a double-strength solution and sterilised by filtration. Both drugs were diluted in the assay medium, dispensed into flat-bottomed microdilution trays (Corning Costar Europe, Badhoevedorp, The Netherlands) and frozen at –70°C until required. The plates contained 100 µL of two-fold serial dilutions of the drugs. Two drug-free medium wells for growth and sterility controls were used.

Inoculum suspensions were prepared by covering the surface of *Aspergillus* colonies with 5 mL of sterile water containing Tween-20 0.1% v/v and probing with a sterile loop. The conidia were transferred to a sterile tube, shaken vigorously, and then adjusted by microscopic enumeration with a cell-counting haemocytometer to provide a suspension of 1×10^6 to 5×10^6 conidia/mL [4]. These suspensions were diluted 1:10 in water, and each well was inoculated with 100 µL of the corresponding conidial inoculum suspension.

CLSI reference method

Susceptibility testing was performed by following the CLSI M38-A guidelines [3]. Dilutions of both antifungal agents were prepared with RPMI-1640 medium that was buffered to pH 7.0 with morpholinopropanesulphonic acid. The drug dilutions were dispensed into round-bottomed microdilution plates

(Corning Costar) and frozen at –70°C until required. The conidial inoculum suspensions were adjusted to an optical density of 80–82%, and diluted 1:50 in RPMI; each well was then inoculated with 100 µL of the corresponding suspension.

Endpoint determination by EUCAST and CLSI procedures

MIC endpoints were defined for both drugs as the lowest drug concentration that caused 100% growth inhibition. The MIC readings were performed with the aid of a concave mirror after incubation for 48 h at 35°C. All experiments were repeated twice on different days.

Analysis of results

Both on-scale and off-scale results were included in the analysis. The reproducibility between the EUCAST and CLSI results was calculated by determining the percentage of agreement between MICs. Agreement was defined as discrepancies between MICs of no more than ± 2 dilutions. In addition, the correlation between the two sets of results was calculated by using the intra-class correlation coefficient (ICC), which was expressed to a maximum value of 1 with a 95% CI. In order to approximate a normal distribution, the MICs were transformed to \log_2 values, with $p < 0.01$ considered to be of statistical significance. The ICC is a reverse measurement of the variability in the counting values. All statistical analyses were performed with SPSS software v.13.0 (SPSS Inc., Madrid, Spain).

RESULTS

MIC data

The MIC ranges and geometric means obtained by the CLSI and EUCAST methods for posaconazole and voriconazole against 40 *Aspergillus* spp. isolates are summarised in Table 1. All the isolates tested grew well both in round-bottomed (CLSI) and flat-bottomed (EUCAST) microtitre plates, giving detectable endpoints within 48 h. A broad range of on-scale MICs was observed with both antifungal agents. *A. niger* was the most susceptible species, while *A. flavus* had higher

Species	Method	MIC (mg/L)			
		Posaconazole		Voriconazole	
		Range	Geometric mean	Range	Geometric mean
<i>A. fumigatus</i>	CLSI	0.015–1	0.08	0.06–1	0.52
	EUCAST	0.03–1	0.12	0.25–2	0.66
<i>A. flavus</i>	CLSI	0.015–0.25	0.11	1–2	1.36
	EUCAST	0.125–1	0.24	1–2	1.74
<i>A. niger</i>	CLSI	0.015–0.25	0.04	0.03–2	0.48
	EUCAST	0.015–0.25	0.08	0.125–2	0.57
<i>A. terreus</i>	CLSI	0.015–0.125	0.04	0.5–1	0.90
	EUCAST	0.06–0.125	0.08	1–2	1.36

Table 1. Posaconazole and voriconazole susceptibilities of 40 isolates of *Aspergillus* spp. as determined by the CLSI and EUCAST broth microdilution methods

CLSI, Clinical Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing.

geometric mean MICs of both voriconazole and posaconazole than did the other species. Discrepancies between the two methods were reflected mainly in higher MIC values obtained by the EUCAST method compared with the CLSI method. However, the average increment was only about a two-fold dilution and was statistically insignificant.

Agreement between the methods

The percentages of agreement and ICC values between the two methods for each *Aspergillus* sp. and both antifungal agents are summarised in Tables 2 and 3. Overall agreement was 92.5% by drug and by organism. Thus, the general levels of agreement were high, and were similar for the different *Aspergillus* spp. tested, although a lower level of agreement was observed for *A. flavus*. Overall, the ICCs were also high (> 0.90), and were statistically significant for all *Aspergillus* spp. On analysis by antifungal agent, voriconazole showed better agreement than posaconazole.

DISCUSSION

This study reports a comparison between the MICs obtained by the CLSI and EUCAST methods for voriconazole and the new triazole posaconazole with clinical isolates of *Aspergillus* spp. The proposed EUCAST standard for testing *Aspergillus* spp. is a microdilution method, based on

the CLSI reference method for antifungal susceptibility testing of filamentous fungi, but includes modifications such as a higher inoculum size and glucose concentration. Denning *et al.* [5] demonstrated that inoculum sizes higher than those proposed by the CLSI M38-A document (1×10^4 to 5×10^4 conidia/mL) generate reproducible in-vitro susceptibility data for *Aspergillus* spp., and were able to identify in-vitro test conditions that consistently differentiated *Aspergillus* strains resistant *in vivo* from susceptible strains. The former strains had high itraconazole MICs, and no benefit was obtained in a murine model, compared with untreated controls, when mice were treated with different doses of itraconazole. Gomez-Lopez *et al.* [6] did not find significant MIC differences using RPMI or RPMI supplemented with glucose, and MIC values were not elevated falsely, even if an inoculum size of 10^5 conidia/mL was used. Other studies have reported no effect on the growth rate of *Aspergillus* spp. when RPMI is supplemented with glucose 2% w/v, even after incubation for 100 h [8].

With respect to inoculum preparation, a previous study in three laboratories [4] evaluated the counting of conidia in a haemocytometer as a means of inoculum preparation for susceptibility testing of *Aspergillus* spp., and was able to demonstrate that inoculum preparation by this method was an accurate and universal procedure, independent of the colour and size of conidia. Moreover, this method was more reliable and reproducible than preparation by spectrophotometric adjustment at 530 nm [9].

Nevertheless, minor variations in test parameters can have a significant influence on MIC values, and should be analysed in detail before implementation in routine clinical laboratories. Previous reports have shown that modifications did not have a significant influence on MICs of amphotericin B or itraconazole [10], indicating that MICs obtained by the CLSI reference method are comparable with those achieved by the proposed EUCAST standard method. MICs obtained in the present study by the EUCAST method were consistently, but not significantly, higher than MICs obtained by the CLSI method, which might be explained by the larger inoculum size used in the EUCAST method. Even so, agreement and concordance between the two methods were high and statistically significant. Thus, the results of

Table 2. Agreement and intra-class correlation coefficient (ICC) between the CLSI and EUCAST broth microdilution methods for different *Aspergillus* spp.

Species	% Agreement	ICC (90% CI)
<i>A. fumigatus</i>	97.5	0.94 (0.89–0.97)
<i>A. flavus</i>	87.5	0.92 (0.85–0.96)
<i>A. niger</i>	90.0	0.93 (0.87–0.96)
<i>A. terreus</i>	93.3	0.97 (0.94–0.98)

CLSI, Clinical Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing.

Table 3. Agreement and intra-class correlation coefficient (ICC) between the CLSI and EUCAST broth microdilution methods for posaconazole and voriconazole

Antifungal agent	% Agreement	ICC (95% CI)
Posaconazole	86.3	0.82 (0.73–0.87)
Voriconazole	98.8	0.90 (0.83–0.93)

CLSI, Clinical Laboratory Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing.

this work demonstrate that susceptibility results obtained by the two methods are comparable, and that the EUCAST method for susceptibility testing of *Aspergillus* spp. is potentially a reliable technique for use in the routine clinical laboratory. Multicentre studies should be performed to confirm these results.

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