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LETTER TO THE EDITOR

Amphotericin B and voriconazole susceptibility profiles for the *Fusarium solani* species complex: comparison between the E-test and CLSI M38-A2 microdilution methodology

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Introduction

Fusarium spp. are ubiquitous fungi that are widely distributed in soil, different organic substrates and plants. They are important pathogens and are responsible for significant economic loss. These fungi are also increasingly associated with human disease and now represent the second most frequent cause of invasive mould infection in immunosuppressed patients [1]. The genus *Fusarium* contains a large number of species, and the most common human pathogens belong to the *Fusarium solani* species complex (FSSC). They are isolated in approximately half of reported infections and cause high morbidity and mortality. *Fusarium* spp. are resistant in vitro to many antifungals; amongst them, FSSC is considered to be the least susceptible [2]. The management of fusariosis is still not well defined; antifungals alone or in combination with other measures, such as surgery or colony-stimulating factors, have been used to treat these infections. Today, according to Nucci and Anaissie, high-dose amphotericin B, combined or not with voriconazole, and reduction of underlying immunosuppression are recommended to treat invasive fusariosis [1]. The aim of this study was to evaluate the in vitro activity of amphotericin B and voriconazole, the most commonly used therapeutics in clinical practice, against a panel of clinical and environmental strains of FSSC. In addition, both methods of susceptibility testing were compared. The Clinical and Laboratory Standards Institute (CLSI) M38-A2 method is considered the reference method [3] and the E-test is a technique often used in medical laboratories.

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Materials and methods

Strains

Forty-eight isolates of FSSC were used in this study. These strains originate from patients, plants and the environment. All strains were deposited at the CBS-KNAW Fungal Biodiversity Centre (http://www.cbs.knaw.nl). Isolates were characterised by molecular typing. The reference isolate, *Fusarium solani* ATCC MYA3636, was included as a quality-control isolate for both the CLSI and E-test methods.

Antifungal agents

E-test (Biomerieux, Marcy l'Etoile, France) gradient strips of amphotericin B and voriconazole were used. They were

stored at −20°C until the day on which the test was performed. The concentration gradient for each drug ranged from 0.004–32 μg/mL.

Amphotericin B (Sigma-Aldrich, Saint Quentin Fallavier, France) powder and voriconazole (Pfizer, Groton, CT, USA) were provided by the manufacturers as assay powders. As described in the CLSI M38-A2 protocol [3], stock solutions were prepared in dimethyl sulphoxide and stored at −20°C. Further dilutions were made in RPMI-1640 medium to yield two times the final concentration (0.03–16 μg/mL) required for the test.

Inoculation preparation

Inocula suspensions were prepared, as described in the CLSI M38-A2 document, from 7-day-old cultures grown on potato dextrose agar slants at 35°C for 72 h and then at 25°C for 96 h. Suspensions of conidia were spectrophotometrically adjusted to optical densities ranging from 0.15 to 0.17 at 530 nm, to obtain 0.4×10^4 to 5×10^4 CFU/mL as the inocula. The final sizes of the stock-inocula suspensions were tested by quantitative colony counts on Sabouraud dextrose agar. The inocula for the conidial suspensions were diluted to 1/50 in NaCl for testing by the CLSI M38-A2 method.

CLSI broth microdilution method (M38-A2 document)

Each microplate well (final volume of 200 μL), containing 100 μL of the diluted drug concentration, was inoculated with 100 μL of the diluted inocula of conidial suspensions. Growth and sterility controls were included for each isolate tested. *Fusarium solani* ATCC MYA3636 was tested as a reference control at each day of testing. Microdilution plates were incubated at 35°C and examined 48 h later for minimal inhibitory concentration (MIC) determination. The MICs were determined by a visual read of complete growth inhibition. All isolates were tested in duplicate using this method of susceptibility testing.

The E-test procedure

The E-test was performed following the manufacturer's instructions. Each solidified RPMI medium (depth 4 mm; AES, Bruz, France) was inoculated by dipping a sterile swab into the respective undiluted inocula suspension and streaking this in three directions over the entire surface of the plate. The agar surface was dried for 15 min, and the strips were placed onto the inoculated agar. The plates were incubated at 35°C, and MICs were determined after an incubation of 48 h. The MICs determined by the E-test were the lowest drug concentrations at which the border of the elliptical inhibition intercepted the scale on the antifungal strip. *Fusarium solani* ATCC MYA3636 was tested as a reference control on each day of testing.

Data analyses

The MICs and MIC ranges determined by the E-test and the CLSI M38-A2 method, the MIC_{90} (cumulative MIC for 90% of isolates tested) and the corresponding GM (geometric mean) values were obtained for each drug tested (Table 1). In order to compare the two susceptibility methods, we determined the percentage agreement as described by Espinel-Ingroff et al. [4]. According to Espinel-Ingroff et al., because the E-test strips contained a continuous gradient instead of the established twofold drug-dilution schema, MICs determined by the E-test were elevated to the next twofold dilution concentration, which matched the drug dilution schema of the CLSI M38-A2 method [4]. This elevation of MICs in the E-test facilitated comparison and enabled presentation of results. As analysed previously [4], discrepancies between the MIC endpoints of no more than three dilutions were used to calculate the percentage agreement (Table 2).

Results and discussion

Among the population of 48 FSSC tested by the CLSI M38-A2 method, amphotericin B MICs (geometric means: 1.2 μg/mL) were lower than those of voriconazole (geometric means: 4.6 μg/mL; Table 1). CMI (geometric means) obtained on each day of testing for *Fusarium solani* ATCC MYA3636 was 1.7 μg/mL for amphotericin B and 4.4 μg/mL for voriconazole. Between each run and both methods, CMI differences were not higher than one dilution.

The proportion of FSSC considered resistant in vitro to the antifungals could not be determined because no interpretative susceptibility breakpoints have been formally proposed for *Fusarium* in the literature or by pharmaceutical laboratories. Thus, the data obtained were compared with previous studies on drug susceptibilities that used the CLSI method. For voriconazole, the geometric means and MIC ranges (1–16 μg/mL) were similar to those of other studies [5–8], and always had high values, i.e. ≥1 μg/mL, which is considered the epidemiological cut-off value for *Aspergillus fumigatus*. For amphotericin B, the range of MICs (0.125–2 μg/mL) was lower than that of other studies, where maximal MIC was 16 μg/mL [6, 9]; however, Tortorano et al. [10] and other authors [11, 12] have reported very similar data to ours. For amphotericin B susceptibility testing, RPMI media was used as recommended by the

Table 1 MICs for 48 FSSCs determined by the E-test and the CLSI M38-A2 method

	MIC (μg/mL)					
	Amphotericin B			Voriconazole		
	GM	Range	MIC_{90}	GM	Range	MIC_{90}
CLSI M38-A2	1.2	0.125–2	2	4.6	1–16	8
E-test	1.9	0.064–32	16	2	0.25–32	8

FSSC, *Fusarium solani* species complex; MIC, minimal inhibitory concentration; GM, geometric mean; MIC_{90}, cumulative MIC for 90% of isolates tested

CLSI, although this method has been demonstrated by others to present a reduced sensitivity for the detection of resistant strains. However, CMI differences between RPMI and AM3 are limited for FSSC as opposed to *Aspergillus* species [5].

For the FSSC population studied, the E-test MICs were more dispersed with larger MIC ranges compared with the CLSI M38-A2 microdilution method: geometric means (ranges) were 1.9 (0.064–32) and 2 μg/mL (0.25–32) for amphotericin B and voriconazole respectively. Compared with the CLSI reference method, the E-test over-estimated amphotericin B MICs and underestimated voriconazole MICs.

This study compared two methods of susceptibility testing. CLSI M38-A2 microdilution is the gold standard method whereas the E-test is a frequently used technique in clinical practice.

In our study, the number of dilutions between MICs obtained by the E-test and CLSI M38-A2 was determined for each strain, and the percentage agreement was calculated for the population studied. For amphotericin B, this value corresponded to 73%, whereas it was higher for voriconazole, at 92%. To our knowledge, ours is the first assay to test voriconazole using the E-test for a population of FSSC. For amphotericin B, agreement between CLSI and the E-test has been calculated at 40% [4] and at 80% [13]; however, discrepancies between the MIC endpoints were evaluated with two dilutions instead of three for the latter study [13]. Agreement between the CLSI and E-test has been evaluated for other moulds, and it is lower for FSSC than for *Aspergillus* species [4, 13].

According to these data, the question is raised whether the E-test is of value in assessing susceptibility in clinical laboratories. For example, a clinical isolate from our population gave an MIC value of 0.5 μg/mL by the CLSI M38-A2 method and a MIC value of 12 μg/mL with E-test for amphotericin B. With the reference method, the mycologist would have considered this antifungal to be active, whereas an opposite interpretation would have been made with the E-test. In addition to the pharmacokinetic characteristics of the drug in humans, which value is the most predictive of in vivo activity of amphotericin B and for clinical outcome?

According to our results, amphotericin B was the most active drug against FSSC. These observations are consistent with the favourable outcome observed for some patients treated with lipid-based amphotericin B [14]. Voriconazole seems to be less active in vitro, although a recent study has shown efficacy in antifungal treatment for fusariosis [15].

This study shows the difficulties in correlating in vitro susceptibility-testing methods, especially for FSSC compared with other moulds, such as *Aspergillus* spp. Correlations between the in vitro susceptibility data and clinical trials, or outcomes from case reports, are even more difficult to establish. Indeed, several factors are involved, such as the number of patients treated, the role of immune reconstitution and delays in initiating antifungal treatment. For this reason, animal models for invasive fusariosis could be an interesting intermediary between in vitro data and the predictive clinical outcomes for patients.

Table 2 Distribution of differences in MICs and percentage agreement between the E-test and the CLSI M38-A2 method

	Number of isolates for which MICs determined by E-test differed from MICs determined by the CLSI M38-A2 method following dilution							
	>+2	+ 2	+ 1	0	− 1	− 2	< − 2	% agreement
Amphotericin B	13	8	13	9	4	1	0	73
Voriconazole	1	0	3	9	18	14	3	92

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