A comparative study of the disc diffusion method with the broth microdilution and Etest methods for voriconazole susceptibility testing of Aspergillus spp.

M. C. Serrano1, M. Ramírez1, D. Morilla1, A. Valverde1, M. Chávez1, A. Espinel-Ingroff2, R. Claro, A. Fernández3, C. Almeida3 and E. Martín-Mazuelos1*

1Servicio de Microbiología, Hospital Universitario de Valme, Ctra Cádiz s/n, Sevilla 41014;
3Servicio de Bioestadística, Unidad Investigación, Hospital Universitario de Valme, Sevilla, Spain;
2Division of Infectious Diseases, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA 2329-80049, USA

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Objective: The activity of voriconazole against Aspergillus spp. (n = 77) was tested by the Etest, disc diffusion and the NCCLS M38-A methods.

Methods: Four Rhizomucor spp. isolates were included to study the suitability of the three susceptibility testing methods to detect isolates resistant to voriconazole. The disc diffusion method performed on Mueller–Hinton agar (Difco) supplemented with 2% glucose and Methylene Blue (0.5 mg/L) gave zone diameters with very clear border edges.

Results: The disc diffusion method showed excellent correlation with the Etest and the NCCLS methods.

Conclusion: On the basis of our results, it appears that the disc diffusion test is a useful method for testing the activity of voriconazole against Aspergillus spp.

Keywords: antifungals, susceptibility tests

Introduction

Patients immunocompromised due to cancer chemotherapy and many other factors, such as corticosteroids, organ transplant or HIV infection, are predisposed to severe fungal infections.1–3 Although Candida albicans is the most common cause of serious fungal infections, the incidence of invasive fungal infections caused by other species of Candida and filamentous fungi, especially Aspergillus spp., has increased.3–6 Amphotericin B, fluconazole and itraconazole are the primary drugs used for the treatment of infected patients.6,7 However, the treatment of invasive fungal infections remains unsatisfactory.3 At present, alternative drugs with activities against Aspergillus and other filamentous fungi species are becoming available for clinical use, including voriconazole and caspofungin.5,9 Voriconazole is a monotriazolic antifungal agent that has a broad spectrum of activity against yeasts and filamentous fungi.10–22 Previous studies have compared the in vitro activity of these new agents against those of established agents. Currently, there is an approved standard method (M38-A) developed by the NCCLS for evaluating the susceptibilities of filamentous fungi to antifungal agents. This method is labour-intensive and not readily applicable in routine laboratories. Agar-based methods are attractive because of their simplicity and low cost, but they are not widely used for antifungal susceptibility testing. Good correlation with the reference method has been reported for fluconazole against Candida spp.23,24 The new antifungal agent voriconazole has been tested extensively in broth-based procedures, but has not been widely evaluated using agar-based testing methods.7,10,12,15,23–28 These previous studies were mainly carried out in Candida spp., but there are a few susceptibility studies that use agar for filamentous fungi.26 This is the first study to compare the disc diffusion method with the NCCLS reference broth microdilution M38-A and the Etest method for voriconazole susceptibility testing of Aspergillus spp.29

Materials and methods

Filamentous fungal isolates

A total of 77 clinical isolates were tested. These included 26 Aspergillus fumigatus, 21 Aspergillus flavus, 10 Aspergillus terreus, nine Asper-
gillus niger, five Aspergillus nidulans, four Aspergillus flavus, two Aspergillus flavipes and four Rhizomucor spp. These isolates were recovered from clinical specimens received at Valme University Hospital in Seville (Spain) and at the Medical College of Virginia, Virginia Commonwealth University (VA, USA). Identification of each strain was performed using routine mycological techniques. The mould isolates were maintained in sterile water and were subcultured on antimicrobial agent-free potato dextrose agar to ensure viability and purity.

Stock inoculum suspensions were prepared from 7-day-old cultures grown on potato dextrose agar (Difco) following NCCLS guidelines.29 Stock suspensions were adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.11 (80–82% transmittance) and contained conidia or sporangiospores and hyphal fragments. The diluted (two times) inoculum sizes ranged from 0.9 × 10^4 to 4.7 × 10^4 cfu/mL, as demonstrated by quantitative colony count on Sabouraud dextrose agar. The same inoculum was used for both broth and agar methods.

Susceptibility testing

Reference antifungal susceptibility testing was performed by the reference broth microdilution method (BMD) described by the NCCLS.29 Broth microdilution MICs were determined after 48 h of incubation at 35°C. The MIC was defined as the lowest drug concentration that resulted in complete inhibition, or MIC-0. The reference powder voriconazole was obtained from Pfizer.

The Etest method was performed as described previously with RPMI agar with 2% glucose (Izasa, España).26,30 Etest strips with voriconazole concentrations ranging from 0.002 to 32 mg/L were provided by AB Biодisk (Solna, Sweden). The plates were inoculated with the inoculum suspension and incubated at 35°C for 24 h and 48 h. Etest MICs were read where the edge of inhibition ellipse intersected the MIC scale on the Etest strip.

Disc diffusion

Testing of voriconazole was performed as described in NCCLS document M44-P for yeast.31 Voriconazole discs (1 µg; Difco, Oxoid) and Mueller–Hinton agar (Difco) supplemented with 2% glucose and Methylene Blue (0.5 mg/L) were used throughout this study. The plates were incubated at 35°C and read at 24 h. Zone diameters were read at the point where growth decreased abruptly.

Quality control

The following strains recommended in the NCCLS M38-A were tested each time to ensure quality control: Candida parapsilosis 22019, Candida krusei 6258 and Aspergillus flavus 204304.29

Analysis of results

In the Etest method, the trailing growth inside Etest ellipse was ignored, as recommended by the manufacturer. Etest MIC endpoints were raised to the nearest two-fold dilution value that matched the NCCLS concentration ranges to facilitate MIC comparisons by both methods. The diameters of zones around 1 µg voriconazole discs were plotted against 48 h broth microdilution and 24 h and 48 h Etest MICs.

Results and discussion

Susceptibility testing was carried out three different ways, using a broth microdilution reference test and two agar-based methods, Etest and disc diffusion. MICs were determined after 48 h by the reference broth microdilution method, and after 24 and 48 h by Etest. As others have shown, the broth microdilution and Etest produced comparable MICs and a good level of agreement for all Aspergillus spp.26,27,30 The 24 h diameters of zones around the 1 µg voriconazole disc were plotted against 48 h broth microdilution and 24 h and 48 h Etest MICs. The medium employed for the disc diffusion method was Mueller–Hinton agar (Difco) supplemented with 2% glucose and Methylene Blue (0.5 mg/L). This medium is recommended in the document M44-P for disc diffusion susceptibility testing for yeast because of its enhanced growth and simplified reading relative to the broth microdilution method.31 Zone size measurements are subjective, and this adds an important source of variability to the test; however, our isolates showed zone diameters with very clear border edges in the Mueller-Hinton agar. Because we did not have any Aspergillus strains with high voriconazole MICs, we included four Rhizomucor strains in this study to ensure the ability of the disc diffusion method to detect strains with high voriconazole MICs. The regression statistics shown in Figures 1–3 demonstrate the excellent correlation between the 24 h zone sizes compared with the broth microdilution and Etest MICs, and similar correlations were found for both tests. The 48 h zones sizes were also measured, but the correlation with both reference tests (broth microdilution and Etest) was worse (data not shown). No diameter pattern was observed to differentiate the Aspergillus species, and all the isolated showed zones diameters >18 mm for voriconazole disc. Zone sizes limits for quality control strains were within the limits established in the M44-P document.

To our knowledge, this is the first report on voriconazole demonstrating a correlation between the in vitro susceptibility data from broth microdilution reference and Etest methods and the disc diffusion method. On the basis of our results, it appears that the disc diffusion test is a useful method for testing the activity of voriconazole against Aspergillus spp., although additional studies with strains with high voriconazole MICs are necessary, as are multi-
Voriconazole susceptibility testing of *Aspergillus* spp.

**References**


