Susceptibility of 100 filamentous fungi: comparison of two diffusion methods, Neo-Sensitabs and E-test, for amphotericin B, caspofungin, itraconazole, voriconazole and posaconazole

IOANA A. COLOSI*, ODILE FAURE†, BÉRANGÈRE DESSAIGNE†, CÉCILE BOURDON†, BERNADETTE LEBAU†, HORAȚIU A. COLOSI* & HÉRVE PELLOUX†

*Iuliu Hatieganu University of Medicine and Pharmacy, Cluj-Napoca, Romania, and †Laboratoire de Parasitologie-Mycologie, Centre Hospitalier Universitaire, Grenoble, France

We compared the E-test method to that of the Neo-Sensitabs tablet diffusion assay for evaluating the in vitro susceptibility of 100 clinical isolates of filamentous fungi (Aspergillus spp., Fusarium spp., Scedosporium spp., zygomycetes and other molds) to amphotericin B, itraconazole, voriconazole, caspofungin, and posaconazole. We determined the categorical agreement level between E-test minimum inhibitory concentrations (MIC) and tablet end-points, as opposed to the following disagreement parameters: very major error – resistant parameter (R) in E-test and susceptible (S) in tablet; major error – S by E-test and R by tablet; minor error – shifts between S and susceptible dose-dependent (S-DD) or S-DD and R. We also performed linear regression analyses and computed Pearson's correlation coefficients (R values) between the log transforms of MICs and the inhibition zone diameters of the five studied antifungal agents. For itraconazole we obtained 97% categorical agreement and R = −0.727. Categorical agreement for caspofungin and voriconazole was 96% and R = −0.821 and R = −0.789, respectively. For posaconazole the categorical agreement was 94% and R = −0.743. Amphotericin B exhibited a lower degree of agreement (76%, R = −0.672), especially in studies of Aspergillus spp. Our results suggest a potential value of the Neo-Sensitabs assay for in vitro susceptibility testing of molds to itraconazole, voriconazole, caspofungin and posaconazole, while amphotericin B exhibited an overall lower degree of agreement.

Keywords filamentous fungi, Neo-Sensitabs, E-test, susceptibility testing

Introduction

In recent years the incidence of filamentous fungal infections increased significantly due to extensive use of anticancer therapy and increasing numbers of transplant and immunocompromised patients [1–3]. Meanwhile, therapeutic possibilities have expanded similarly due to the development of new antifungal agents (triazoles and echinocandins). In vitro antifungal susceptibility testing of filamentous fungi is requested more often due to the emergence of resistant isolates and to the larger number of available antifungal agents [4].

Intense efforts have been made to achieve a consensus on testing the susceptibility of filamentous fungi to antifungals to establish reproducible and standardized methods [5].

The Clinical and Laboratory Standards Institution (CLSI) broth micro-dilution method for testing of molds (M38-A) is a reference procedure but is difficult to use in clinical laboratories in daily practice [6].

Alternative diffusion methods have been developed in order to evaluate the susceptibility to antifungal drugs, which were inspired by the reference technique (medium, inoculum, interpretative criteria).
E-test (AB Biodisk, Solna, Sweden) is one such method which involves significantly less work than the broth microdilution procedure. Determining minimum inhibitory concentrations (MIC) allows categorizing strains as susceptible (S), susceptible dose-dependent (S-DD), or resistant parameter (R). After numerous comparative studies with reference methods, E-test for molds was found to be simple, reproducible, and suited for routine use in clinical laboratories [7–15].

A new disk diffusion procedure (method M44-A) was proposed by CLSI to evaluate the in vitro susceptibility of yeasts on agar medium (Mueller-Hinton medium supplemented with glucose and methylene blue) using antifungal disks [16]. Several studies [14,17–21] attempted to evaluate susceptibility of filamentous fungi in a similar manner as the one used for yeasts. Depending on the diameter of the inhibition zone, Candida strains were classified as S, susceptible dose dependent (S-DD) or R to tested antifungals.

The agar disk diffusion method could be a simple, flexible and cost-effective alternative to the E-test for use in clinical microbiology laboratories. The aim of our study was to evaluate the E-test and Neo-Sensitabs tablet diffusion assay (Rosco, Denmark) to determine the in vitro susceptibility of filamentous fungi to amphotericin B, caspofungin, itraconazole, voriconazole, and posaconazole.

**Material and methods**

A total of 100 strains of filamentous fungi isolated from clinical samples (e.g., sputa, broncho-alveolar lavages, bronchial aspirates, tracheal aspirates, wounds, biopsy, etc.) of in-patients at the Grenoble University Hospital, France, were identified and evaluated by E-test and Neo-Sensitabs tablet diffusion assay with amphotericin B, caspofungin, itraconazole, voriconazole and posaconazole.

The tested isolates included Aspergillus fumigatus (50), A. flavus (12), A. terreus (eight), A. niger (five), A. nidulans (three), A. glaucus (one), Aspergillus spp. (one), Eurotium spp. (one), Scedosporium spp. (three), Fusarium spp. (five), Absidia spp. (three), Mucor spp. (two), and one strain of Acremonium spp., Alternaria spp., Penicillium spp., Paecilomyces spp., Trichoderma spp., Monilia strophila (strains available for the scientific community). The identification of each isolate was determined on the basis of macroscopic and microscopic characteristics.

The quality control strains Candida krusei ATCC 6258 and C. parapsilosis ATCC 22019 were employed as controls.

The MIC for each antifungal in the E-test was established as the point of intersection of the ellipse representing the inhibition zone and the E-test strip. For amphotericin B, MIC was read as total growth inhibition (100%), whereas with azoles (itraconazole, voriconazole, and posaconazole) and caspofungin the E-test was performed according to the manufacturer’s instructions. The E-test MIC was the lowest drug concentration at which the borders of the elliptical inhibition intercepted the scale on the antifungal strip [9–11].

The inoculum suspension was adapted according to species, between 6 × 10^4 and 9.5 × 10^4 CFU as determined by colony counts on Sabouraud dextrose agar. The growth-medium used was RPMI 1640 supplemented with 2% glucose, incubated at 35°C for 24–48 h.

We used 9 mm antifungal tablets in the Neo-Sensitabs diffusion assays of the following antifungals; amphotericin B 10 μg, itraconazole 8 μg, voriconazole 1 μg, homemade posaconazole disk (Schering-Plough) (sterile disk impregnated with 5 μg/tablet), and caspofungin 5 μg. We impregnated sterile disks (5.35 mm diameter, 30 mg weight) with 20 μl solution containing 250 μg posaconazole/ml. The solution used as solvent was DMSO (dimethyl sulfoxide). Neo-Sensitabs assays were performed according to the manufacturer’s instructions and guidelines for mold testing on non-supplemented Muller-Hinton agar plates [19], with the same inoculum concentrations as for E-test. The Mueller-Hinton agar plates were inoculated as described and tablets were applied. The plates were then incubated at 35°C for 24–48 h. Reading of inhibition diameter zones (mm) was performed at 24 h for zygomycetes and Aspergillus and at 48 h for one strain of A. terreus, A. glaucus, and for all other species of filamentous fungi (due to their slower growth rates).

Zone diameters were measured to the nearest whole millimeter at a point in which there was a prominent reduction of growth (80% for azoles and caspofungin) or no visible growth (100% inhibition for amphotericin B).

**Interpretative criteria**

For Aspergillus species and azoles we used the following criteria [22]:

- For itraconazole: susceptible ≤ 1 μg/ml for A. fumigatus, A. flavus, A. nidulans and A. terreus; and ≤ 2 μg/ml for A. niger;
- For voriconazole: susceptible ≤ 1 μg/ml for A. fumigatus, A. flavus and A. terreus; and ≤ 2 μg/ml for A. nidulans and A. niger;
- For posaconazole: susceptible ≤ 0.5 μg/ml for A. niger and A. terreus; ≤ 1 μg/ml for A. nidulans; and ≤0.25 μg/ml for A. fumigatus and A. flavus.

We considered an isolate with MIC above as being resistant.

For Aspergillus species and caspofungin we used the following criteria [23]:

- Susceptible: ≤ 0.5 μg/ml for A. fumigatus and A. nidulans; ≤ 0.25 μg/ml for A. flavus, A. terreus, and A. niger.
We considered an isolate with MIC above as being resistant.

Since breakpoints for molds have not yet been agreed upon for antifungal agents, we used the following interpretative criteria for isolates other than *Aspergillus*, as well as for *Aspergillus* and amphotericin B [19]: susceptible ≤ 1 μg/ml and ≥ 17 mm (triazoles and caspofungin) or 15 mm (amphotericin B); intermediate/susceptible dose-dependent 2 μg/ml and 14–16 mm (triazoles and caspofungin) or 13–14 mm (amphotericin B); resistant ≥ 4 μg/ml and ≤ 13 mm (triazoles and caspofungin) or ≤ 12 mm (amphotericin B).

**Data analysis**

In our evaluation we determined the categorical agreement level between E-test MICs and tablet end-points, as opposed to the following disagreement parameters: very major error – resistant parameter (R) in E-test and susceptible (S) in tablet; major error – S by E-test and R in tablet; minor error – shifts between S and susceptible dose-dependent (S-DD) or S-DD and R. Statistical analysis was performed using SPSS 13.0 for Windows, by linear regression analysis and the computation of Pearson’s correlation coefficients between the normalized log transforms of MIC endpoints and the inhibition zone diameters of the five antifungal agents.

**Results**

After performing the tablet procedure using plain Mueller-Hinton agar, we observed that inhibition zones were well defined and easy to read (Fig. 1). For the majority of isolates results were obtained after 24 h (48 h for Fusarium, *Alternaria, Paecilomyces* and some strains of *A. fumigatus*). Categorical agreement levels found between E-test and Neo-Sensitabs tablet assay are shown in Table 1.

Categorical agreement for itraconazole between E-test and Neo-Sensitabs in our study was 97%, with two very major errors (one strain of *Mucor* and one strain of *Absidia*) and one major error (one strain of *A. fumigatus*).

The correlation coefficient R between Log2 MICs and the inhibition zone diameters was −0.727 (inversely proportional relation). The linear regression model for itraconazole MIC prediction based on inhibition zone diameters is shown in Fig. 2.

The percentage of categorical agreement between E-test and Neo-Sensitabs for voriconazole was 96%, with one very major error (one strain of *Mucor* spp.), and three minor errors (one strain of *Absidia* and two strains of *Fusarium*).

The correlation coefficient R between MICs and inhibition zone diameters was −0.789 and the linear regression model for MIC prediction based on inhibition zone diameters is found in Fig. 3.

The percentage of categorical agreement between E-test and Neo-Sensitabs for posaconazole was 94%, including three very major errors (one strain of *A. fumigatus*, one strain of *Fusarium*, and one strain of *Absidia*) and three minor errors (one strain of *Trichoderma*, one strain of *Mucor* and one strain of *Acremonium*).

The correlation coefficient between MICs and inhibition zone diameters was −0.743, with the linear regression

---

*Fig. 1* Antifungigrams obtained by E-test (right) and Neo-Sensitabs (left).
model for MIC prediction based on inhibition zone diameters as shown in Fig. 4.

The percentage of categorical agreement between E-test and Neo-Sensitabs for caspofungin was 96% with four major errors (one strain of *Mucor*, one strain of *Acremonium*, one strain of *A. terreus*, and one strain of *A. nidulans*).

The correlation coefficient R between MICs and inhibition zone diameters was \(-0.821\). The linear regression model for caspofungin MIC prediction based on inhibition zone diameters is presented in Fig. 5.

We obtained the lowest categorical agreement between E-test and Neo-Sensitabs assay with amphotericin B, i.e., 76%. There were five very major errors (four strains of *A. flavus* and one strain of *A. terreus*), three major errors (one strain of *A. flavus*, one strain of *A. terreus* and one strain of *A. fumigatus*) and 16 minor errors (five strains of *A. flavus*, two of *A. fumigatus*, two of *A. terreus*, three of *Fusarium*, and one strain each of *Scedosporium*, *Paecilomyces*, *Eurotium* and *Mucor*).

The correlation coefficient R between Log2 MICs and the inhibition zone diameters was \(-0.672\). The linear regression model for amphotericin B MIC prediction based on inhibition zone diameters is provided in Fig. 6. In all above cases the computed R-values exhibited a high statistical significance \((p \ll 0.001)\).

For QC strains, MCIs and inhibition zones values were within the reported limits [24]. The agreement between E-test and Neo-Sensitabs results with these isolates was 100% for *C. parapsilosis* ATCC 22019 for all tested antifungal agents. We noted similar agreement with *C. krusei* ATCC 6258 in studies of caspofungin and azoles, while one minor error was observed for amphotericin B.

**Discussion**

In this study we performed the tablet procedure by following the guidelines [19] for the *in vitro* susceptibility testing of molds using plain Mueller-Hinton agar. Several studies demonstrated that supplemented Mueller-Hinton agar did not support suitable growth of a variety of molds, while

---

**Table 1** Categorical agreement between E-test® and Neo-Sensitabs® tablet assay.

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>Number of errors</th>
<th>% of categorical agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minor</td>
<td>Major</td>
</tr>
<tr>
<td>Posaconazole 5 μg</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Caspofungin 5 μg</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Voriconazole 1 μg</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Itraconazole 8 μg</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Amphotericin B 10 μg</td>
<td>16</td>
<td>3</td>
</tr>
</tbody>
</table>
Fig. 3  Voriconazole – graphical representation of the linear relation between Log2 MIC (E-test®) and inhibition zone diameters (Neo-Sensitabs®).

Fig. 4  Posaconazole – graphical representation of the linear relation between Log2 MIC (E-test®) and inhibition zone diameters (Neo-Sensitabs®).
inhibition zones on plain Mueller-Hinton agar were better defined and easier to read [17,18].

For itraconazole, categorical agreement found by our study (97%) between MICs (μg/ml) and inhibition zones (mm) was higher than agreements reported in previous investigations, i.e., 83% [18] and 85% [19], respectively. Nevertheless, we also observed a number of agreement errors for zygomycetes isolates (Mucor and Absidia, one isolate each), but itraconazole tablets appeared suitable for testing other filamentous fungi.

Like several authors [14,18] who have evaluated the correlation between MICs and inhibition zone diameters for voriconazole, we also found a good correlation between these two test methods ($R = -0.789$). Serrano et al. [14] compared a disk diffusion method with the broth reference and E-test procedures in susceptibility tests with this antifungal using 77 isolates of Aspergillus spp. The authors noted an excellent correlation between 24 h zone sizes and broth microdilution MICs, as well as those obtained with the E-test.

Espinell-Ingroff and Canton [19] evaluated for the first time the performances of the Neo-Sensitabs tablet assay in studies of voriconazole with molds and reported that the percentage of categorical agreement was 90%. Agreement errors were found among Fusarium spp., Alternaria spp., and B. spicifera, but the use of voriconazole tablets was able to identify resistant strains. The authors concluded that the tablet assay could be a good choice for in vitro susceptibility studies of molds to voriconazole.

Several studies [17–21] have evaluated the performance of a disk diffusion assay for susceptibility testing of filamentous fungi to posaconazole. All showed a good correlation between disk/tablet diffusion assay and broth microdilution method and an excellent level of categorical agreement (>90%). In our study of posaconazole, the percentage of categorical agreement between E-test and Neo-Sensitabs was 94%, with agreement errors among Fusarium, zygomycetes and Acremonium isolates. Other studies [18,19] also have reported agreement errors among Fusarium and zygomycetes isolates, but the method was able to detect resistant isolates.

The performance of Neo-Sensitabs tablet assays of caspofungin against mold isolates was evaluated for the first time by Espinel-Ingroff and Canton in 2008 [19]. The authors concluded that the tablet assay ‘could be the choice’ for testing susceptibility of molds to caspofungin (95% overall categorical agreement). As shown in a previous investigation [18], we also found errors among A. nidulans isolates, but the caspofungin tablets were able to identify resistant strains.

We obtained the lowest categorical agreement between E-test and Neo-Sensitabs assay (76%) in tests of amphotericin B and a lower correlation level between Log2 MICs and inhibition zone diameters. Previous studies [17–19]...
have also demonstrated lower correlation levels between amphotericin B Log2 MICs and inhibition zone diameters. The majority of categorical agreement errors found in our investigation of amphotericin B were among *Aspergillus* spp. (mainly *A. flavus*) and *Fusarium* isolates.

The main disadvantages of Neo-Sensitabs are the lack of MIC values and lack of interpretive zone diameters for all antifungal agents. Based on the diameter of the inhibition zone there is a possibility of MIC prediction by carrying out linear regression models.

The regression analyses in our study were not influenced by the values of breakpoints (the R-values and the equations of the regression models did not depend on the breakpoints). We tried to show that if the inhibition zone diameter (mm) is known, the MIC can be estimated based on regression analysis.

In conclusion, Neo-Sensitabs tablet diffusion assay for evaluating the *in vitro* susceptibility of filamentous fungi to antifungal agents (especially for triazoles and caspofungin) may represent a cost-effective and simple alternative to E-tests for routine application in clinical laboratories.

The diameters of the inhibition zones obtained by Neo-Sensitabs tablet assay were well defined and easy to read, proving the Neo-Sensitabs method to be quick and simple to use. The Neo-Sensitabs tablet assay demonstrated a good ability to identify resistant isolates.

Categorical agreement between Neo-Sensitabs and E-test for caspofungin and azoles (itraconazole, voriconazole, posaconazole) were excellent. All zygomycetes are resistant to caspofungin, as well as to a large extent to voriconazole, so there is no real need to test these two antifungal agents. The only limit of Neo-sensitabs concerns amphotericin B which would appear to be less suitable for testing *Aspergillus* isolates.

**Acknowledgements**

We thank Schering-Plough for providing posaconazole pure drug.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**References**

Susceptibility of filamentous fungi: comparison of two diffusion methods

This paper was first published online on Early Online on 6 October 2011.