Comparison of two methods, E-test® and Neo-Sensitabs® tablet diffusion assay, for testing susceptibility of 93 Candida strains to amphotericin B, fluconazole, voriconazole, and caspofungin

Compararea două, E-TEST și testul de difuzie NeoSensitas pentru detectarea infecției a 93 de tulpini de Candida la amfotericină B, fluconasol, voriconazol și caspofengin.

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ABSTRACT

Objectives: Antifungal susceptibility testing of Candida species is increasingly requested due to the large number of antifungal agents now available and the emergence of resistant isolates. Neo-Sensitabs® tablet diffusion assay is easy to perform and seems reliable in routine susceptibility testing of yeasts to antifungal agents. We compared this method with the E-test® procedure, by testing 93 strains of Candida spp.

Methods: We tested 93 strains of Candida (Candida albicans 45 strains, Candida glabrata 21 strains, Candida tropicalis 8 strains, Candida krusei 6 strains, Candida parapsilosis 5 strains, Candida kefir 4 strains, Candida lusitaniae 3 strains and Candida guillermondii 1 strain) from clinical samples of Grenoble Hospital inpatients. Two Quality Control strains (C. parapsilosis ATCC 22019, C. krusei ATCC 6258) were included as controls.

Neo-Sensitabs® tablet diffusion assay (Rosco) uses 9 mm diameter tablets for all antifungal agents (amphotericin B 10µg, fluconazole 25µg, itraconazole 8µg, voriconazole 1µg, caspofungin 5µg). Mueller-Hinton agar supplemented with 2% glucose and 0.5 µg/ml methylene blue (BioMérieux) was used. The E-Test® method (AB Biodisk) was performed in accordance with the manufacturer’s instructions, with E-test® strips for all antifungal agents and RPMI agar. Inoculum was equivalent to 0.5McFarland standard, between 1x10^6 and 5x.10^6 CFU/ml and plates were incubated at 35ºC for 24-48 h.

We determined categorical agreement levels between E-test® MIC and tablet end-points, as opposed to the following disagreement parameters: very major discrepancies R to E-test® and S to tablet; major discrepancies S to E-test® and R to tablet; minor discrepancies- shifts between S and SDD or SDD and R.

Statistical analysis was performed using linear regression analyses and Pearson’s correlation coefficients (R values) between the log transforms of MICs and the inhibition zone diameters of the antifungal agents.

Results: We obtained 100% (R=-0.654), 100% (R=-0.62), and 98.92% (R=-0.679) of categorical agreement for fluconazole, voriconazole and caspofungin respectively. Amphotericin B exhibited a lower degree of agreement with 90.32% of categorical agreement (R=-0.622).

In all cases, the above computed R-values exhibited a high statistical significance (p<<0.001).
Conclusion: The results of our study suggested a potential value of the Neo-Sensitabs® assay for testing fluconazole, voriconazole, and caspofungin, while amphotericin B exhibited an overall lower degree of agreement.

Key words: Candida, E-test®, Neo-Sensitabs®, antifungal agents.

Introduction
In recent years the incidence of fungal infections, especially Candida infections (candidiasis), increased significantly due to enlarged use of anticancer therapy and increasing numbers of transplanted and immunocompromised patients (21, 22). Concurrently, the therapeutic range has expanded along with the emergence of new antifungal agents (triazones and echinocandin) (3, 15). Laboratory aid in the choice of therapy is particularly important in specifying the susceptibility of Candida to various antifungal drugs. Intense efforts have been made to achieve a consensus on testing of yeasts susceptibility to antifungal agents, and establishing reproducible and standardized methods for testing susceptibility and their correlation to clinical results (4, 5, 7, 26).

NCCLS (CLSI) broth micro-dilution method for testing susceptibility to antifungal agents of yeasts (M27-A2) is a reference method difficult to use in laboratories for daily practice (3). The use of this method also has revealed its limits: difficulty to identify strains of Candida with reduced susceptibility to amphotericin B, phenomenon of residual growth observed for the azoles and insufficient growth of some Candida species (13, 18, 23, 25).

Alternative methods have been developed to test the susceptibility to antifungal drugs (disk diffusion methods), methods that were inspired by the reference technique (medium, inoculum, interpretative criteria), to which they were compared (6, 11, 12, 17, 27).

E-test® (AB Biodisk, Solna, Sweden) is an alternative method for testing antifungal susceptibility which involves much less work than broth microdilution method. Determining minimum inhibitory concentrations, (MIC), allows categorizing strains as susceptible (S), intermediate (I) or resistant (R) to tested drugs. After numerous investigations and studies, E-test® proved simple, standardized, suited for routine use in clinical laboratories, reproducible and even more reliable than the reference technique for detecting resistance to amphotericin B of Candida spp. (2, 6, 9, 13, 18, 23, 24, 25).

Disk diffusion procedure with new identified guidelines (method M44-A) was recently proposed by CLSI to evaluate susceptibility of yeasts on agar medium (Mueller-Hinton medium supplemented with glucose and methylene blue) using antifungal discs (5). Depending on the diameter of the inhibition zone, Candida strains were classified as S, susceptible dose dependent (SDD) or R to tested antifungals. Although this standardized method is currently available only for fluconazole and voriconazole, several studies have been performed in order to set or improve standards for other antifungal agents such as caspofungin, anidulafungin, micafungin, amphotericin B, posaconazole, itraconazole (11, 12).

Since it could be important to find alternatives with similar advantages, the aim of our study was to perform a comparison between E-test® and Neo-Sensitabs® tablet diffusion assay (Rosco, Denmark) for testing susceptibility of Candida species to antifungal drugs.

Material and methods
Ninety three strains of Candida isolated from clinical samples (bronchopulmonary and tracheal aspirates, sputa, broncho - alveolar lavage fluids, hemocultures, catheters, gastric and peritoneal fluids, urines, and secretions from wounds) of Grenoble (France) Hospital inpatients were identified and evaluated by E-test® and Neo-Sensitabs® tablet diffusion assay.

Candida strains were identified by using Candida ID 2 medium (bioMerieux, France), Glabrata RTT®, and Krusei-Color® (Fumouze), and ID 32C strips (bioMerieux).

The quality control strains Candida krusei ATCC 6258 and Candida parapsilosis ATCC 22019 were also used as controls.

The tested species were Candida albicans (n = 45), Candida glabrata (n = 21), Candida tropicalis (n = 8), Candida krusei (n = 6), Candida parapsilosis (n = 5), Candida kefyr (n = 5).
4), Candida lusitaniae (n = 3) and Candida guilliermondii (n = 1).

Susceptibility of Candida strains was tested to the following antifungals: voriconazole, itraconazole (only for Candida krusei strains instead of fluconazole), fluconazole, amphotericin B, and caspofungin.

The first method used was the E-test®: MIC was determined for each antifungal at the point of intersection of the ellipse representing the inhibition zone and the E-test® strip.

Inoculum: several well-isolated colonies from a 24 to 48 hours pure culture on Sabouraud dextrose agar were homogenized in 0.85% NaCl to obtain a turbidity equivalent to 0.5McFarland standard (corresponding to a suspension of 1-5x10⁶ CFU). The growth-medium used was RPMI 1640. A sterile swab was soaked into the suspension and then the agar plates were inoculated using Retro C80™ (rot-a-plater, AB BIODISK) by streaking the entire surface. After drying for 10-15 minutes, E-test strips were applied using a specific device. Incubation period was 24-48 hours at 35 ° C.

For amphotericin B, MIC was read for a total inhibition (100%) growth of Candida. For azoles (voriconazole, fluconazole, itraconazole) and caspofungin MIC was read at a growth inhibition of 80%, according to the manufacturer’s instructions.

According to the MIC value obtained for each antifungal agent, Candida strains were classified as S, SDD / I or R (Table 1) (4, 19, 20).

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>S (susceptible) µg/ml</th>
<th>SDD (susceptible dose-dependent)/ I (intermediate) µg/ml</th>
<th>R (resistant) µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>voriconazole</td>
<td>≤ 1</td>
<td>2</td>
<td>≥ 4</td>
</tr>
<tr>
<td>fluconazole</td>
<td>≤ 8</td>
<td>16-32</td>
<td>≥ 64</td>
</tr>
<tr>
<td>itraconazole</td>
<td>≤ 0,125</td>
<td>&gt; 0,125 and &lt; 0,5</td>
<td>≥ 1</td>
</tr>
<tr>
<td>amphotericin B</td>
<td>≤ 1</td>
<td></td>
<td>≥ 2</td>
</tr>
<tr>
<td>caspofungin</td>
<td>≤ 1</td>
<td></td>
<td>&gt; 2</td>
</tr>
</tbody>
</table>

The second method used was Neo-Sensitabs® tablet diffusion assay using Mueller-Hinton medium supplemented with 2% glucose (providing adequate growth of yeasts) and 0.5 mg/ml methylene blue (providing a better definition of the inhibition zone diameter), with 9 mm antifungal tablets: amphotericin B 10 µg, fluconazole 25 µg, itraconazole 8 µg, voriconazole 1 µg, and caspofungin 5 µg. Neo-Sensitabs® assay was performed according to the manufacturer’s instructions and M44-A guidelines (5).

The same inoculum as for E-test® was used. Supplemented Mueller-Hinton agar plates were inoculated as described and 9 mm tablets were applied. The plates were then incubated at 35°C. 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) after 24 – 48 hours (Figure 1). According to the inhibition zone diameter, Candida strains were classified as S, SDD / I or R (Table 2) (5).

<table>
<thead>
<tr>
<th>Antifungal agent</th>
<th>S (susceptible) mm</th>
<th>SDD (susceptible dose-dependent) mm</th>
<th>R (resistant) mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>voriconazole 1 µg</td>
<td>≥ 17</td>
<td>16 - 14</td>
<td>≤ 13</td>
</tr>
<tr>
<td>fluconazole 25 µg</td>
<td>≥ 19</td>
<td>18 - 15</td>
<td>≤ 14</td>
</tr>
<tr>
<td>itraconazole 8 µg</td>
<td>≥ 15</td>
<td>14 - 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>amphotericin B 10 µg</td>
<td>≥ 15</td>
<td>14 - 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>caspofungin 5 µg</td>
<td>≥ 15</td>
<td>14 - 12</td>
<td>≤ 11</td>
</tr>
</tbody>
</table>
Results and discussion

We determined categorical agreement levels between E-test® MICs and tablet endpoints, as opposed to the following disagreement parameters: very major discrepancies R to E-test® and S to tablet; major discrepancies S to E-test® and R to tablet; minor discrepancies - shifts between S and SDD or SDD and R.

Statistical analysis was performed using linear regression analyses and Pearson’s correlation coefficients (R values) between the log transforms of MICs and the inhibition zone diameters of the antifungal agents. For linearization of MICs (measured on an exponential scale) the logarithm of this variable was used. Regression models were obtained using SPSS 13.0 for Windows.

The following results were obtained (Table 3):

<table>
<thead>
<tr>
<th>Antifungal agents</th>
<th>Method</th>
<th>Number of isolated species</th>
<th>Number of discrepancies</th>
<th>% of categorical agreement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S  I  R</td>
<td>Minor errors</td>
<td>Major errors</td>
</tr>
<tr>
<td>amphotericin B</td>
<td>E-test®</td>
<td>83  2  8</td>
<td>6  0  3</td>
<td>90.32%</td>
</tr>
<tr>
<td></td>
<td>Neo-Sensitabs®</td>
<td>88  4  1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fluconazole</td>
<td>E-test®</td>
<td>86  0  1</td>
<td>0  0  0</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Neo-Sensitabs®</td>
<td>86  0  1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>voriconazole</td>
<td>E-test®</td>
<td>93  0  0</td>
<td>0  0  0</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>Neo-Sensitabs®</td>
<td>93  0  0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>caspofungin</td>
<td>E-test®</td>
<td>91  1  1</td>
<td>0  1  0</td>
<td>98.92%</td>
</tr>
<tr>
<td></td>
<td>Neo-Sensitabs®</td>
<td>90  1  2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For amphotericin B the categorical agreement between E-test® and Neo-Sensitabs® assay was 90.32%. Six minor disagreements (5 strains of C. glabrata and 1 strain of C. albicans), and 3 very major discrepancies (2 strains of C. glabrata and 1 strain of C. krusei) were observed. The correlation coefficient R between Log2 MICs and the inhibition zone diameters was -0.622 (Figure 2).
Acquired resistance of *Candida* species to amphotericin B is rare. Increases in MIC to amphotericin B have been described among *C. lusitaniae*, *C. krusei* and *C. glabrata* (21). Acquired resistance is associated with alterations of the contents of cellular membrane lipids, particularly sterols. ERG3 defects in the gene involved in synthesis ergosterol causes the accumulation of other sterols in the fungal membrane. Resistant strains of *Candida* are lower in ergosterol compared with sensitive strains (21).

In our study, 8 *Candida* strains were resistant to amphotericin B with E-test®: 5 strains of *C. glabrata*, 2 strains of *C. krusei* and 1 strain of *C. albicans*. With Neo-Sensitabs® assay only 1 strain of *C. krusei* was R, and 1 strain of *C. glabrata* was intermediate susceptible (minor error). Two strains of *C. glabrata* were intermediate susceptible (E-test®) and S to Neo-Sensitabs® diffusion assay (minor discrepancy).

In different previous studies in which Neo-Sensitabs® tablets and other diffusion methods were evaluated (11, 12) the level of categorical agreement between MICs and inhibition zone diameters ranged from 98.2% to 98.9% and the R values from 0.47 to 0.48.

For fluconazole, categorical agreement found by our study between E-test® and Neo-Sensitabs® was 100%. The correlation coefficient R between Log2 MICs and the inhibition zone diameters was -0.654 (Figure 3).

Figure 2. Amphotericin B – graphical representation of the linear relation between Log2 MIC (E-test®) and inhibition zone diameters (Neo-Sensitabs®).
Some strains of *C. glabrata* may be SDD to fluconazole and up to 22% of strains may be R (21). Some strains of *C. tropicalis*, *C. norvegensis*, *C. dubliniensis*, and *C. inconspicua* may express occasionally an increased MIC to fluconazole (21).

In our study, only 1 strain of *C. albicans* was resistant to fluconazole (E-test® and Neo-Sensitabs®). The overall MIC 90 for fluconazole was 2µg/ml.

In a previous study that evaluated Neo-Sensitabs tablets® (11) the R value was 0.88 and the percentage of categorical agreement was 95.5%.

For voriconazole, the percentage of categorical agreement between E-test® and Neo-Sensitabs® was 100% in our study. All *Candida* strains were susceptible to voriconazole. All *C. krusei* strains and *C. albicans* strain that were resistant to fluconazole were also susceptible to voriconazole. The correlation coefficient R between MICs and inhibition zone diameters was -0.62 (Figure 4).
Another study (11) found the R value for voriconazole 0.79 and the percentage of categorical agreement between MICs and inhibition zone diameters 95.5%.

Similar to another study (20), MIC 90 of voriconazole for *C albicans* in our study (0.023 µg/ml) was lower than MIC 90 for *C glabrata* (0.094 µg/ml). Two strains of *C. glabrata* had MIC=0.25 µg/ml.

In our study, only the 6 strains of *Candida krusei* were tested against itraconazole, with the following results: E-test® - all strains were SDD (MIC> 0.125 µg/ml); Neo-Sensitabs® - all strains were susceptible. Since the number of isolates tested against itraconazole in our study was small we cannot state any conclusion regarding the behavior of itraconazole Neo-Sensitabs® tablets.

Due to the phenomenon of residual growth observed for azoles, the inhibition zone on E-tests® was not always well delimited, so MICs were sometimes difficult to read. Meanwhile, the inhibition zone diameters were well defined and easy to read.

For caspofungin, the percentage of categorical agreement between E-test® and Neo-Sensitabs® was 98.92%. One major discrepancy was found (1 strain of *Candida krusei* S by E test® and R by the tablet method). One strain of *Candida glabrata* was R (non-susceptible) by both methods, with a MIC> 32 mg/ml respectively, an inhibition zone diameter of 0 mm. The correlation coefficient R between MICs and inhibition zone diameters was -0.679 (Figure 5).

**Figure 4.** Voriconazole – graphical representation of the linear relation between Log2 MIC (E-test®) and inhibition zone diameters (Neo-Sensitabs®).
In another study that evaluated tablets of caspofungin (9 mm diameter, 5μg, Rosco) (11) the R value was 0.82 and the percentage of categorical agreement between MIC and inhibition zone diameter was 94.5%.

As in a previous studies (8, 19), the MIC values for *C. krusei* and for *C. parapsilosis* in our study (0.25-0.5μg/ml) were higher than the MICs for *C. albicans* (0.016-0.125μg/ml). The MIC for *C. guilliermondii* was 1.5μg/ml.

**Conclusions**

E-test® is a standardized, easy to use method. MIC values (μg/ml) are sometimes difficult to read and the method is expensive (25 euro/Candida strain).

Categorical agreement between Neo-Sensitabs® and E-test® for voriconazole and fluconazole was 100%. Categorical agreement for caspofungin was excellent, as well (98.92%). A lower categorical agreement with E-test® was found for amphotericin B (90.32%).

Infection with *C. glabrata* in patients who were under echinocandin therapy has been described (9, 10, 14, 28). Echinocandin resistance has also been reported in patients with *C. albicans* infection (1, 16). *Candida* species resistance is associated with point mutations in the gene complex Fks1 synthesis coding β-1, 3-D glucan (9, 21).

In all cases above the computed R-values exhibited a high statistical significance (p<<0.001).

The diameters of the inhibition zones are well defined and easy to read, proving the Neo-Sensitabs® method to be simple to use. Also, the cost of Neo-Sensitabs® is five times lower than for E-tests® (5 euro/Candida strain).

The main disadvantage of Neo-Sensitabs® consists in the lack of MIC values. Also, interpretive zone diameters are not yet available for all antifungal agents.

Given the above, Neo-Sensitabs® tablet diffusion assay for testing susceptibility to antifungal agents of *Candida* species may
represent a cost-effective and simple alternative to E-tests® in clinical laboratories. Based on the diameter of the inhibition zone, there is a possibility of MIC prediction by carrying out linear regression models. Such regression models have been proposed for amphotericin B, fluconazole, voriconazole, and caspofungin.

**REZUMAT**

**Obiective:** Testarea sensibilității la antifungice a tulpinilor de *Candida* este esențială datorită apariției unor tulpinii de *Candida* rezistente la antifungice și a creșterii numărului de antifungice. Metoda difuzimetrică de testare a sensibilității la antifungice a tulpinilor de *Candida* utilizând tablete de 9 mm Neo-Sensitabs® (Rosco) pare o metodă de încredere și ușor de utilizat în practica zilnică în laboratoarele clinoce. Am comparat această metodă cu o altă metodă de testare a sensibilității la antifungice, E-testul® (AB Biodisk), testând 93 tulpini de *Candida*.


**Rezultate:** Procentele concordanțelor calitative obținute și valorile coeficientelor de corelație R au fost de 100% (R=0,654), 100% (R=0,62) și respectiv 98,92% (R=0,679) pentru Fluconazol, Voriconazol și Caspofungină. Pentru Amfotericină B procentul concordanțelor calitative a fost mai scăzut 90,32%, iar valoarea lui R a fost de 0,622. În cazul tuturor antifungicelor corelațiile au fost semnificative statistic (p<0,001).

**Concluzii:** Rezultatele studiului nostru sugerează că metoda difuzimetrică pentru testarea sensibilității la antifungice a speciilor de *Candida* utilizând tablete de antifungice de 9 mm diametru Neo-Sensitabs® pentru Fluconazol, Voriconazol și Caspofungină poate reprezenta o alternativă la E-test® în laboratoarele clinice. Nivelul de concordanță dintre cele două metode a fost mai scăzut pentru Amfotericină B.

**Cuvinte cheie:** *Candida*, E-test®, Neo-Sensitabs®, antifungice.

**References**


